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Development of disease and drug models for rare diseases: Application to Acute Intermittent Porphyria

Diego Vera Yunca

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Development of disease and drug models for rare diseases: Application to Acute Intermittent Porphyria

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"People's dreams don't ever end!"

- Marshall D. Teach

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Preface

Rare diseases represent a challenge for drug discovery and development due to the low number of patients eligible for clinical trials in addition to the other factors affecting drug development in general. Although regulatory agencies encourage the pharmaceutical companies to focus on rare diseases, those are not as attractive as other diseases such as cancer or neurological diseases. Computational modeling and simulation in the pharmacological setting, known as pharmacometrics, represents a great opportunity to integrate sparse information from *in vitro*, *in vivo*, and clinical data in order to better understand the disease mechanisms and how treatments restore body homeostasis. Through model simulations, current and new therapies can be explored *in silico* to determine the best pharmacodynamic targets, experimental designs, dosing regimens, and combination strategies among others, thus making drug research and development programs more efficient and reducing attrition rates.

This thesis illustrates an evolving mechanistic framework developed for Acute Intermittent Porphyria (AIP) at the discovery and pre-clinical stages of development. This type of approach provides quantitative support for already established mechanisms of the biological system involved, allows the search for new pathways, and incorporates drug effects on the specific target(s). We believe that the information gathered during the present investigation will help the research and development of innovative therapies for AIP and beyond. The current thesis has been organized as follows:

The *Introduction* section briefly describes rare diseases and the challenges of designing clinical trials for this kind of diseases. Then, it is focused on the different modeling approaches applied to rare diseases caused by a genetic mutation along the gene expression process, ending with some considerations for the use of modeling in translational approaches from preclinical to clinical studies.

Chapter 1 presents to the best of our knowledge the first computational model developed for AIP mice. The urinary excretion of heme precursors, the biomarkers of AIP, in porphyric mice during phenobarbital-induced acute attacks were well described by the proposed disease progression model which infers the heme biosynthesis pathway and the processes occurring in liver and blood. Model parameters were accurately estimated, and part of the data was used to validate externally the model. A theoretical simulation was performed by adding the effect of the standard-of-care for AIP, hemin, to demonstrate the potential capabilities of this mechanistic framework

Chapter 2 expands this mechanistic modeling framework by including data from an innovative therapy, a mRNA encoding a human porphobilinogen deaminase (PBGD), which is the mutated

enzyme in AIP. The kinetics of the different mRNA formulations, the dynamics of the heme precursors and the effects of the exogenous PBGD on restoring normal levels of these biomarkers were adequately predicted by the model. Data came from different animal species, and several mRNA formulations were investigated allowing the estimation of formulation- and animal-specific parameters, which facilitated the projections of mRNA formulations to humans in a search for an optimal dosing scenario.

Chapter 3 shows the most developed AIP model up to date. It described well the urinary excretion of the heme precursors over time for control mice and for those that were treated with a new recombinant PBGD modified to target the liver. The fact that the experimental setting included three different formulations, several dose levels and the intravenous and subcutaneous routes of administration allowed the characterization of regulatory mechanisms that remained hidden during the previous analyses described in *chapters 1* and *2* above, such as the inhibitory effects of heme on the ALA synthase enzyme at the start of the heme biosynthesis pathway, or the different molecular processes that cause acute attack induction by phenobarbital. The protective effects of the different recombinant PBGD formulations were evaluated by simulating different experimental scenarios in which the therapies were administered at different times prior to the induction of the AIP attack.

The *General Discussion* section highlights the main aspects of the three chapters and integrates them with the considerations considered in the *Introduction* section. Finally, the last section, *Conclusions*, presents a summary of the main results of this thesis.

As part of the education training program, during the course of this thesis, an eight-month research stay at the Merck Institute for Pharmacometrics (Lausanne, Switzerland), was performed. The goal was to acquire experience in applying pharmacometrics in the pharmaceutical industry, and to gather expertise in other therapeutic areas by handling large datasets where new approaches as machine learning might have an impact in the development of more predictive population pharmacokinetic/pharmacodynamics models. The research focus was on oncology, specifically the application of a new methodology to characterize tumor heterogeneity and developing tumor growth inhibition and overall survival models that included innovative predictors based on genetic mutations, location of the primary lesion and tumor heterogeneity. The results of this work were published and are included as appendices in this thesis.

Introduction

Drug discovery and development is a lengthy and complex process. One out of 1000 compounds is authorized to be tested in clinical trials after undergoing the pre-clinical stage, and then only 10% of those are approved after finalizing the clinical phases of development. Moreover, this process can last up to 15 years and can cost more than $1 billion from the first stages of drug discovery until market authorization (1). This process is even more difficult for rare or orphan diseases. There is not a universal definition for a rare or orphan disease. In the United States, it is defined as a disease which affects a population up to 200,000, whereas the Committee for Orphan Medicinal Products of the European Medicines Agency states that a rare disease is a serious, life-threatening condition with a prevalence up to 5 per 10,000 (2).

The low prevalence of rare diseases poses as an important issue for drug research and development. Pharmaceutical companies are not as interested in developing therapies for these diseases as for other major conditions such as cancer, infectious diseases or cognitive disorders (e.g. Alzheimer's disease, Parkinson's disease). In return, regulatory agencies encourage the research and development of new therapies for rare diseases by offering incentives both in the European Union (3) and in the United States (4) such as reduced regulatory fees, longer market exclusivity after drug approval and research grants.

Enrollment in clinical trials is hampered by the reduced number of patients in the target population (5). For example, cystic fibrosis is one of the most known rare diseases (6), yet the prevalence is only 1 in 1400 people to 1 in 3500 people (7). Besides, most of the rare diseases have less than ten conducted clinical trials, while the six most known ones (all related to rare types of cancer) have more than a thousand trials (8). To overcome the issues derived from a small sample size in clinical trials, other kinds of study designs could be used, such as crossover trials, N-of-1 trials or adaptive design (9). These strategies require a specific statistical analysis in order to calculate the required sample size or to evaluate the results of a trial when there is not enough statistical power (10).

Modeling in drug development

While clinical data for rare diseases are limited, preclinical data (i.e. *in vitro* or animal *in vivo* models) are not as scarce (2). The real challenge is to integrate all existing data for a given rare disease, regardless of its origin, to maximize the potential results of data analyses. Leveraging all the information can lead to a better knowledge of disease mechanisms and drug effects. Modeling and simulation in the field of pharmacometrics is one of the best approaches to handle all the data simultaneously and in a quantitative way. In fact, drug regulatory agencies are actively promoting the use of Model-Informed Drug Discovery and Development (MID3) to support pre-clinical and clinical studies. United States' Food and Drug Administration (FDA) launched a Critical Path initiative in 2004 to modernize drug discovery and development, encouraging the development

of quantitative disease models to improve the design of future clinical trials and to better select clinical outcome metrics (11). Furthermore, a Model-Informed Drug Development pilot program was created to promote a more widespread application of modeling techniques and to further inform regulatory decisions (12). These approaches are also encouraged by the European Medicines Agency (EMA). The Modeling and Simulation Working Party supports and informs the Committee for Medicinal Products for Human Use and other EMA's scientific groups, promoting further integration of MID3 in the standard drug marketing authorization application (13). Pharmacometric reviews derived from MID3 have supported the approval of drugs for special patient groups (e.g., pediatric populations) or in situations where the approved drug dose was not tested in the clinical trial itself (14).

Drug research and development driven by model-based approaches have been extensively described (15). As an example, oncology is one of the areas not exempted for challenges and limitations that have been extensively studied under pharmacometrics umbrella. Cancer clinical trials present several challenges, such as the lack of a placebo arm in most cases, the limited number of drug doses tested and a large risk of drug toxicity (16). To overcome these issues, pharmacometrics plays a key role of in oncology (17) and its popularity has led to the development of different computational models that can describe different kinds of cancer data (18). Another important therapeutic area for pharmacometrics is antibiotics in order to design the best dosing regimens for special populations, such as critically ill patients (19), by using data from preclinical *in vitro* and *in vivo*. Several models have been developed to explain bacterial growth, drug effect on bacterial death and bacterial resistance to antibiotics (20).

In contrast, computational modeling of rare diseases is not a widespread practice yet. There are several works describing potential modeling approaches for rare diseases using *in silico* (21) approaches to find the best drug, and the number of filings for orphan drugs including model-based analyses have increased over the last years (22). However, the number of pharmacometrics-oriented scientific papers found in literature is limited when compared to the large number of described rare diseases. Most of those works focus on empirical, descriptive models driven by the data (build following the "top-down" approaches) such as pharmacokinetic (PK) models(23–25) predicting concentration-time profiles or pharmacokinetic-pharmacodynamic (PK-PD) models (26,27) linking drug exposure to the measured response over time by using straightforward PD models such as the sigmoidal maximal effect (Emax) model (28). These models allow researchers and companies to estimate accurate model parameters and to predict exposure and effect metrics in certain constrained scenarios to design the best dosing schedule; however, the knowledge gained on the disease progression and the mechanisms of action of the treatments is limited.

The opposite approach is to build very complex models that include every known component, mechanism or reaction related to the drug exposure, its effect and/or the disease itself (i.e. "bottom-up" approaches) (29). Examples of this kind of models are quantitative systems pharmacology (QSP) models and physiologically-based pharmacokinetic models (PBPK). All the information collected in preclinical and clinical stages is leveraged, therefore the knowledge about a given rare disease and its treatment can be expanded and extrapolated to other species or patient groups. This kind of model is oriented towards reproducing biological systems accurately and discovering new therapeutic targets rather than towards obtaining precise and identifiable model parameters.

An intermediate approach is to build mechanistic models that include key components of the disease, drug exposure and therapeutic effects while being able to identify model parameters ("middle-out" approaches) (30). These models allow to explore and confirm molecular, genetic or feedback mechanisms related to the pathophysiology of the disease while delivering reliable results than can be reproduced or used to perform clinical trial simulations. Its reduced complexity also shortens computational run times when compared to full systems biology models. Mechanistic models are a great opportunity for rare disease researchers to integrate known processes such as mutations impairing enzymes, regulatory feedbacks and chemical reactions occurring in the metabolic or signaling pathways related to the disease. Figure 1 displays an overview of the different modeling approaches.

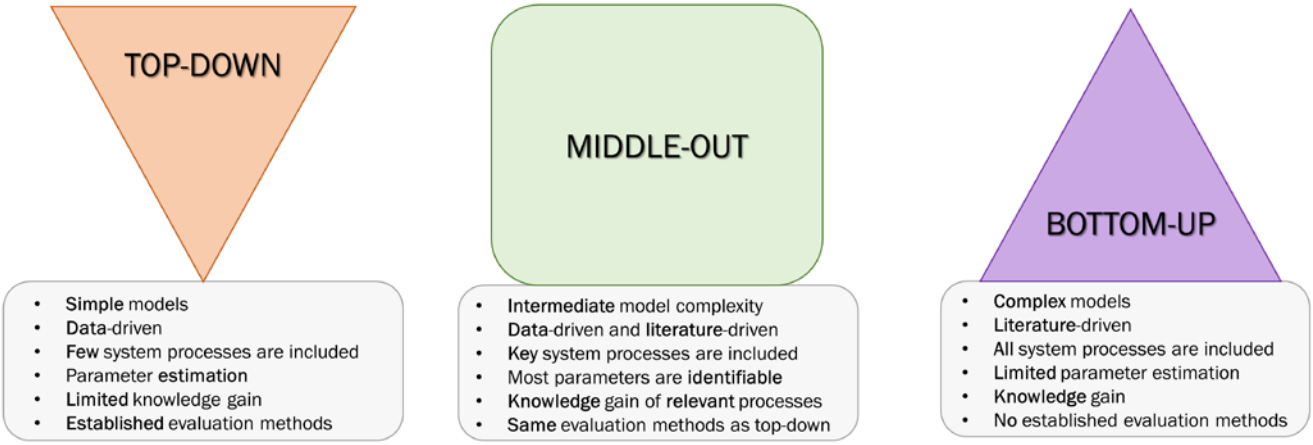

Figure 1. Overview of the different modeling approaches to inform drug discovery and development.

Modeling approaches such as "middle-out" can help to better understand and to better treat rare diseases, especially those which are caused by a genetic mutation that causes a loss-of-function or a gain-of-function protein, such as an enzyme or a transporter. In these diseases, there are several steps of the pathophysiology that can be potential targets for different kinds of treatments and therapies. In a classic gene expression process, the gene is transcribed into messenger RNA,

which in turn is translated into the final protein. This protein exerts its effect or action (e.g. to catalyze a chemical reaction, to transport molecules) that generates a final product or effect which is measured as the response (although the protein itself may be considered as the final outcome). All these components of the gene expression process are susceptible of being inhibited or changed with a treatment or therapy, and model-informed drug development can play a key role in improving preclinical or clinical trials regardless of the kind of drug and target which is analyzed

In this review we aim to evaluate the different model approaches (either top-down, bottom-up or middle-out) carried out in rare diseases along the whole gene expression process, from the gene itself till the protein that causes the disease's final outcome, as shown in Figure 2.

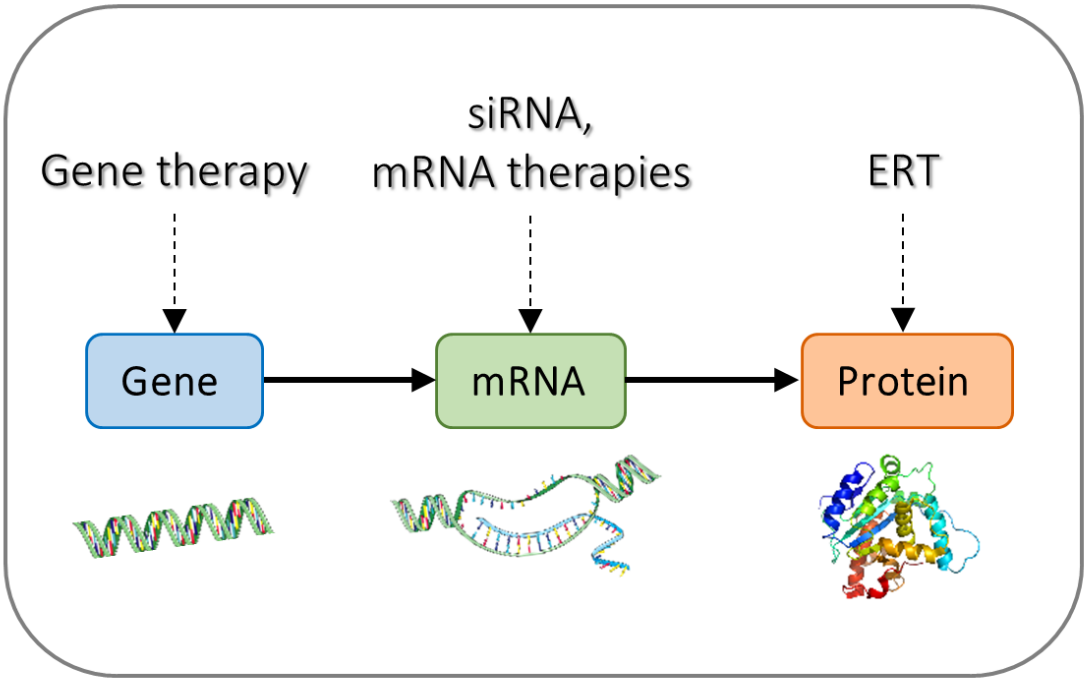

Figure 2. Gene expression process: targets for current and potential therapies in rare diseases. siRNA, small interference ribonucleic acid. mRNA, messenger ribonucleic acid. ERT, enzyme replacement therapy.

Modeling and simulation in the gene expression process

Gene and DNA-based therapies

The mutated gene itself can be targeted or replaced using gene therapy. The most innovative strategy up to this day is gene editing using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system (31), which is able to insert gene fragments into the host's genome. This allows either to silence genes by producing untranslatable or non-functional proteins, or to repair mutated genes by introducing the right sequence instead

of the erroneous gene. Other gene therapies do not target the host's gene, instead they provide non-integrating vectors that can be translated and traduced into the desired protein by the cellular machinery (32).

As these therapies use DNA or RNA sequences that are susceptible of being degraded by nucleases, delivery vectors are used to protect the exogenous genetic material and to direct those to the target tissues. Non-viral vectors have been explored (33), but nowadays i*n vivo* gene therapies often utilize recombinant viral vectors. One of the most used virus for gene delivery is the recombinant adeno-associated virus (rAAV). This virus does not contain gene sequences of itself, but genomes which express the therapeutic proteins (34) once the virus has successfully infected a cell.

Modeling and simulation efforts in the gene therapy area are scarce, although some works have described either the pharmacokinetics or the pharmacodynamics of the delivered exogenous gene sequences (35). The first developed models for gene therapy were aimed at describing systemic pharmacokinetics of DNA linked to cationic surfactants (36,37) using simpler population PK models. A semi-mechanistic model was developed to explain the intracellular kinetics and transcription of a plasmid DNA under different experimental conditions. Model results showed the existence of two DNA forms with different transcription properties, and the suppression effect of different molecules on the DNA transcription to mRNA (38). More complex computational models have been built, such as the one by Varga *et al.* covering most of the processes and mechanisms occurring during the internalization, endosomal escape and nuclear import of the DNA sequence to form the encoded protein. By characterizing all the transfection processes, a model-based exploration allowed to identify the slowest, rate-limiting reactions which could be potentially improved. This assessment showed that the rAAV vector was the most efficient over non-viral vectors. The exposure of a rAAV-delivered interleukin-12 (IL12) DNA and its relationship with interferon-γ was characterized by building a target-mediated drug disposition model (39), which displays the binding mechanisms and kinetics of both compounds and their respective receptors (40). This model successfully and simultaneously characterized the kinetics of IL12, interferon-γ, and their interaction.

Messenger RNA (mRNA) and RNA-based therapies

The erroneous gene sequence is transcribed into messenger RNA (mRNA). This molecule is susceptible of being blocked by the RNA interference mechanism, which uses small interference RNA (siRNA) sequences to target and silence specific mRNA strands (41). In rare diseases,

siRNA is used to avoid the translation of the mutated gene transcript or proteins related to rate-limiting steps that must be stopped.

The use of model-based approaches for siRNA therapies is limited. Most pharmacometric analyses are empirical, focused on systemic siRNA exposure and utilize straightforward methods such as non-compartmental PK analyses (42) or simple compartmental models (43,44).

An example of a rare disease treated with siRNA is acute intermittent porphyria (AIP). It is a rare metabolic disease caused by a loss-of-function genetic mutation in the third enzyme of the heme biosynthesis pathway, porphobilinogen (PBG) deaminase (PBGD). The lack of PBGD activity increases the amounts of PBG and 5-aminolevulinic acid (ALA) in liver during acute porphyric attacks, leading to an accumulation of these heme precursors in blood. The excess of ALA and PBG is thought to be the main cause of the symptoms during an acute attack (45). Hemin arginate is the standard-of-care therapy for AIP patients suffering acute attacks, although its administration presents several side effects (46). This represents an opportunity for the development of new therapies. One of those innovative drugs is a siRNA which targets the ALA synthase 1 (ALAS1) mRNA. ALAS1 is the first and most important enzyme of the heme pathway. The inhibition of its translation reduces the amount of ALA synthesized in liver (and consequently, the amount of PBG derived from ALA (47)). This siRNA has been already approved as a drug therapy (givosiran) for AIP patients by the FDA (48) and EMA (49), and PK parameters such as the apparent central volume of distribution and the apparent clearance were determined by using a population PK model (48). For the EMA submission (49), two computational models were presented: (i) a population PK-PD model that linked givosiran systemic exposure to its effect (modeled as a sigmoidal inhibitory maximal effect function or Imax (50)) on urinary ALA excretion and (ii) a model which coupled urinary ALA levels and the incidence of porphyric attacks.

Another potential approach is to introduce the mRNA molecule itself into the cells. This would lead to the translation of a non-mutated, functional protein that would not cause the disease. Therapies based on mRNA have become famous nowadays because of the COVID-19 pandemic mRNA-based vaccines (51), but mRNA treatments are also used for other diseases such as cancer (52) or rare diseases (53). As with gene therapy, mRNA therapies are often encapsulated (formulated) to avoid degradation and to direct the molecules towards the target tissue (54).

Modeling works for mRNA-based treatments are still uncommon, and most are focused on the estimation of PK parameters for product approval by the regulatory agencies. Nevertheless, there are models that describe both the PK and PD of mRNA therapeutic molecules. For example, the work by Almquist *et al*. (55) shows the systemic exposure and the effects of a mRNA encoding vascular epithelial growth factor A (VEGF-A) on wound healing. Degradation of mRNA, protein

synthesis and its elimination over time were characterized using simple compartmental models (a one-compartment model for mRNA and an indirect responses (56) model for VEGF-A, respectively), while the VEGF-A effect on the wound healing process (described using a logistic growth expression) is included as an Emax model. Model-based simulations describing other experimental scenarios can be performed in order to find, in this case, the optimal dose to achieve a faster wound healing with a smaller dose level. Research articles displaying population approach works of mRNA therapies in rare diseases are even rarer. Our group developed a mechanistic PK-PD model able to characterize the urinary excretion of heme precursors in AIP animal models after the administration of an mRNA which encodes the human PBGD (hPBGD) enzyme (57). This work is presented in *Chapter 2* of this thesis. First, the release and degradation processes of mRNA were defined (the latter being formulation-specific), and the translation to the final protein in the liver was considered proportional to the amount of free mRNA in the organ. Then, measured urinary amounts of heme precursors were considered proportional to their counterparts in liver, where the hPBGD exerts its effect by decreasing ALA and PBG amounts and by increasing the transit to porphyrins (and consequently, to heme). PBGD activity at baseline presented different values depending on the animal species. This allowed to integrate data from different sources regardless of the species, and to then extrapolate this model to humans.

Protein and enzyme-based therapies

The protein is the final molecule of the gene expression process. It is the responsible for the final response that is measured, or it is the final outcome by itself. Most classical therapies for rare diseases caused by a defective protein (e.g. an enzyme) are aimed at either replacing the protein with a functional one (58) or blocking the faulty molecule to avoid its harmful effects (59). As these treatments have been used in preclinical, clinical trials and in clinical practice for years or even decades, the majority of computational models describing the exposure and the effect of rare disease therapies are focused on protein-based therapies.

Several rare diseases treated with enzyme replacement therapy (ERT) or similar approaches have been studied by building non-linear mixed effects models. Growth hormone deficiency (GHD) is a rare disease that causes short stature and other growth disorders (60). The main treatment for this disease is the daily subcutaneous or intramuscular administration of human growth hormone (hGH) in order to restore its levels in the body. New formulations of hGH aim to release the drug in a sustained way, thus reducing the number of administrations. The work presented by Fisher *et al*. showed a PK-PD approach to characterize the systemic exposure and the effect of the long-acting hGH formulation MOD-4023 on insulin-like growth factor 1 (IGF-1) levels. A two-compartment model best fitted the observed MOD-4023 concentrations in serum, while IGF-1 levels were modeled using an indirect responses model. MOD-4023 increased IGF-1 synthesis,

and this effect was included into the model as an Emax equation. Data from the recombinant hGH (r-hGH) formulation were also modeled using a one-compartment model. Then, model simulations displayed the longer residence time in serum for the innovative MOD-4023 formulation compared to the r-hGH one. The most important application derived from this modeling work was the determination of peak and mean levels of a derived IGF-1 safety-related biomarker by using model simulations.

Mucopolysaccharidosis Type VII is another rare disease, a lysosomal storage disorder caused by the lack of β-glucuronidase (GUS) enzymatic activity needed to degrade glycosaminoglycans (GAGs) (61). Qi *et al*. developed a model to describe the concentration-time profiles of Vestronidase alfa, a recombinant human GUS, and its effect on decreasing GAGs excreted in urine. A two-compartment model with linear elimination was selected as the best disposition model for Vestronidase alfa. Then, the best or optimal dosing regimen was determined by performing model simulations. Area under the curve (AUC) for Vestronidase alfa was computed using the PK model in order to link AUC values to the observed urinary GAG values. A sigmoidal inhibitory function or Imax connected individual AUC values with the observed decrease in urinary GAGs, allowing not only to predict and simulate Vestronidase alfa exposure but its effect on restoring the normal GAG levels.

Another rare lysosomal storage disorder is Gaucher disease (GD). In this case, the impaired enzyme is glucocerebrosidase (GBA1). The deficiency of this protein lead to the accumulation of glucosylceramide in macrophages, leading to their transformation in Gaucher cells (62), especially in bone marrow, spleen and liver. The most common kind of GD is Type 1 (90% of all GD cases) (63). A semi-mechanistic population PK model proposed by Gras-Colomer *et al*. (64) was built to predict the glucocerebrosidase kinetics in plasma and leukocytes with a time-dependent clearance, allowing for therapy individualization in patients with GD Type 1. The work by Abrams *et al*. (65) followed another modeling approach: a QSP model ("bottom-up" approach) which incorporated all the relevant processes and components of the macrophages, plasma and liver. This model was able to include the two current kinds of treatments for GD: ERT and substrate reduction therapy. After calibrating the model, it could be used to explore how the different processes behave when the system was perturbed. This model also provided answers to more clinical questions such as the effects of switching therapies or the behavior of a more heterogeneous GD population.

The third rare lysosomal storage disorder we explored looking for modeling approaches is acid sphingomyelinase deficiency (ASMD). As its name says, it leads to a reduced acid sphingomyelinase activity, which in turn produces the accumulation of sphingomyelin and similar molecules in macrophages and hepatocytes, causing tissue damage (66). Kaddi *et al*. (67)

developed a complex QSP model which reflected the key processes occurring in the system of ASMD patients, such as (i) a PBPK model for the ERT olipudase alfa (recombinant human acid sphingomyelinase) and submodels at the (ii) molecular, (iii) cellular and (iv) organ levels. The final QSP model was used to simulate different clinical scenarios at short or long term, such as different dose values for patients with different ASMD severities.

Hemophilia is a group of inherited rare coagulation disorders in which the blood does not clot properly. This is caused by the lack of either factor VIII (hemophilia A, the most common type) or factor IX (hemophilia B) (68). The severity of the disease depends on the residual factor VIII or IX activity in blood (69). A population PK model was developed by Garmann *et al*. (70) for a recombinant human factor VIII therapy. This model took into account the presence of concentration values below the limit of quantification (BLQs) for parameter estimation, as the final values (e.g. terminal half-life) were overestimated when BLQs were excluded from the model. Abrantes *et al*. (71) took a step further and built a model which connected systemic factor VIII exposure and bleeding frequency, which is the main outcome of patients with hemophilia A. To achieve this goal, a repeated time-to-event (in this case the event is bleeding) model was created to predict the probability of the bleeding event to happen and how factor VIII concentrations reduced this probability. This model was able to simulate the cumulative number of bleeds that a given patient would suffer depending on his factor VIII activity, thus physicians could optimize factor VIII doses to reduce bleeding.

AIP, as described before, is a rare disease which shows a decreased PBGD activity causing the accumulation of heme precursors ALA and PBG. *Chapter 3* of this thesis shows how a mechanistic modeling approach was able to predict ALA and PBG levels in urine for control and treated AIP mice and to account for the most important regulatory mechanisms in the disease pathway. Data from control AIP mice were used to describe the disease dynamics before, during and after several acute attacks. A new recombinant PBGD ERT therapy targeted to liver restored the heme biosynthesis pathway and reduced ALA and PBG levels in blood and especially in liver. The large number of different PBGD formulations and dose values improved the characterization of all the disease- and drug effect-related processes. A simulation exercise was performed to determine the best dosing regimen (i.e. route of administration, number of doses and dose value) depending on the time of administration with respect to the acute attack. The “middle-out” approach that was applied meant that most parameter values were obtained by following a data-driven approach, model structure was built by taking into account the actual components of the heme biosynthesis pathway and the different organs or tissues.

Modeling in rare diseases: from preclinical to clinical studies

As its name suggests, a rare disease presents a limited number of patients. Clinical studies for rare diseases are challenging to organize and manage. Patients are scarce and the number of experts who know about the rare disease are limited and usually focused on a specific aspect of the disease. The few patients eligible for a given clinical trial have to travel long distances to the treatment sites, which reduces even further the actual number of study participants. Besides, most of the information about a rare disease often comes from a small number of clinical case studies (5). These issues make preclinical drug development in rare diseases even more important before starting clinical trials. By efficiently using data from *in vitro* studies and animal models, we can characterize the drug kinetics and its effects. Moreover, we can gain knowledge of the disease mechanisms and processes that can inform and optimize dosing decisions or even give birth to new, innovative therapies. Figure 3 summarizes the model-guided process from preclinical experiments to clinical trials.

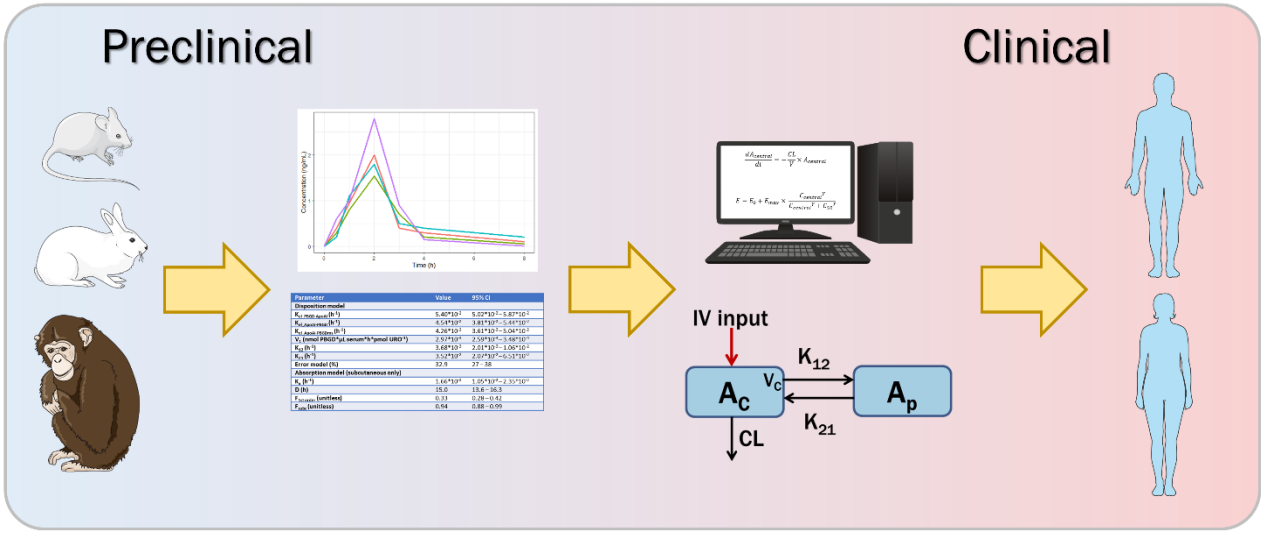

Figure 3. From animals to humans: model-guided drug development. Animal models provide preclinical data and information that can be modeled *in silico*. These preclinical models are then extrapolated by scaling key model parameters in order to obtain human predictions**.**

In order to quantitatively describe a disease by using model-based approaches, longitudinal data is needed. This means that preclinical experiments must be designed in a way that allows the researcher to collect as many observations or values over time as possible while taking into account experimental limitations in the quantification techniques or in the cellular or animal models. Longitudinal measurements enable the modeler to estimate key parameter values *in silico* (e.g. synthesis or degradation rate constants) that otherwise would have been obtained after performing one or several experiments. An important concept for these mechanistic preclinical models is perturbation of the system. This means that, in order to know more about the dynamics of a disease, the system (i.e. animal, *in vitro* cell) must be altered from the baseline status in as many different ways as possible: by using different formulations of the same drug or even

different drugs, different doses and dosing times. By performing these experiments, the disease is being perturbed from different angles, allowing to characterize processes that otherwise would remain hidden. We encourage to carry out experimental designs that take into account this perturbation concept in order to provide more rich data for executing better model-based analyses.

In the absence of longitudinal data or when the number of assessments is very low, previous knowledge about the disease or similar ones have to be applied to the model. When considering this previous information, it must be stated that assumptions are considered when building a model, leading to potential limitations. These suppositions can overcome the existing gaps in knowledge, guide new experiments and validate hypothesis.

There are some cases when there is no information at all to introduce in our model: this means that there is no experimental data or previous literature in any form to base the initial parameter value on. To overcome this issue, a multidisciplinary team is a key factor. Clinical experts must be consulted whenever possible to discuss whether the proposed parameter value or mechanism is clinically and physiologically plausible. After the approval by the clinical team, the model must be calibrated to find the parameter value that improves the model the most, while keeping the clinical significance of the parameter (i.e. clinically unreal parameter values will not be considered during the model building process).

Once the final model has been defined and all parameter values have been either estimated or fixed based on literature or model calibration, it can be used to explore different aspects of the pathophysiology. The most common simulations include several dosing scenarios such as different dose values, routes and times of administration to optimize dosing regimens for future preclinical trials. Nevertheless, other kinds of simulations unrelated to the therapeutic drug and focused on the disease model can be run. For example, if you have modeled a rare disease by using data from urine and you have included liver compartments, you could assess unmeasured hepatic dynamics *in silico* for the unperturbed and treated conditions. Such models do not only provide answers to treatment-related questions, but they also offer insights into disease processes that might inform of new or poorly-understood mechanisms.

Computational models developed for preclinical data can be extrapolated to predict and simulate scenarios for other species, including humans. To achieve this, several model parameters must be allometrically scaled, as physiological features (e.g. baseline enzymatic concentrations, organ volumes) and the differences in parameters accounting for synthesis and degradation must be considered. First, it must be determined which parameters need to be scaled, as many of the disease and drug processes may show the same parameter values shared across species. Then, the scaling factor for each species must be obtained, either from experimental values or literature. Allometric scaling is usually performed by taking into account body weight or age. Once the

appropriate parameter values have been set, model simulations for the different species can be performed to, for example, find the optimal dosing regimen in humans using data from mouse, rat or other animal model. This can inform the first steps of clinical trials, e.g. first-in-human phase I clinical studies.

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Aims

Drug discovery and development in rare diseases is a challenge that needs support from other fields. Pharmacometrics can be a useful ally to leverage knowledge of the disease leading to more efficient development programs applying the MID3 paradigm.

The general aim of this thesis was to develop mechanistic and translational models for the rare disease Acute Intermittent Porphyria (AIP), to be applied in case of innovative therapeutics and other rare diseases, mainly those involving heme-related hepatic enzymopenias. This general aim includes the following specific objectives:

1. To develop a semi-mechanistic perturbation/disease model that describes the urinary excretion of heme precursors (5-aminolevulinic acid [ALA], porphobilinogen [PBG] and porphyrins) in AIP mice during acute attacks induced by phenobarbital administration. This model framework represents the basis for future AIP model extensions that include effects elicited by therapeutics.

2. To integrate new data across different species from a new messenger RNA (mRNA) therapy encoding for the human PBG deaminase (PBGD) in a computational model framework characterizing the kinetics of mRNA in liver, the dynamics of the transcription and protein expression processes, and their effects on the fate of heme precursors during an acute attack. A key deliverable of this modeling exercise is the projection of the efficacy to support the development of clinical programs.

3. To expand the previous AIP disease and drug effect models leveraging data obtained from a developing program of new recombinant PBGD fusion proteins targeted to liver. Likely, this modeling exercise will end into a model in which key processes of the heme pathway, otherwise hidden, are characterized.

Overall, the conjunction of data, assumptions on the different mechanisms of the biological system, and decisions taken during the model building process across the different experimental settings aim to impact the development programs beyond the current investigational therapeutics and the particular case of acute intermittent porphyria.

Chapter 1

Computational disease model of phenobarbital-induced acute attacks in an acute intermittent porphyria mouse model.

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Chapter 2

Disease pharmacokinetic-pharmacodynamic modelling in acute intermittent porphyria to support the development of mRNA-based therapies.

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Chapter 3

Mechanistic modelling of enzyme-restoration effects for new recombinant liver-targeted proteins in acute intermittent porphyria

Submitted

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General discussion

The current research efforts in rare diseases are limited. The nature of this area i.e., low number of patients and the existence of heterogeneous disease presentations (different clinical and/or genetic manifestations (1)) makes the development of new therapies and the design of clinical trials not as attractive for pharmaceutical companies as for other diseases such as the cancer or neurological diseases. Despite this fact, many research groups keep pushing for new discoveries in the rare diseases area. Most of those efforts have been carried out in the *in vitro* and *in vivo* pre-clinical arena. The resulting data must be efficiently analyzed to make the most of this basic research, and pharmacometrics represents an excellent niche for leveraging information from different sources to better understand the rare disease and to provide tools for a better design of clinical trials and a better development of new, innovative therapies.

A variety of different modeling approaches and efforts used to describe treatment effects and disease progression in rare diseases have been identified. Despite the rise of the physiological-pharmacokinetic based modelling in drug development and model-based precision dosing, most of the examples in rare diseases fall into the category of empirical data-driven models ("top-down" approach) developed to simply describe systemic exposure. An intermediate mechanistic modeling approach would be the development of a partially physiological pharmacokinetic (PK) model that characterizes the drug kinetics in blood and in the target organ, for example liver in hepatic diseases. With respect to treatment effects, several works related systemic exposure with clinical response using pharmacodynamic models. Certainly, multiscale mechanistic models pooling data from different experimental sources to characterize the disease at molecular, cellular and organ level (bottom-up approach) are challenging in rare disease as data and knowledge is still very scarce from a quantitative system pharmacology perspective. Therefore, the "middle-out" (2) approach appears as an appealing alternative and it represents the strategy adopted through all the analysis presented in this thesis.

The University of Navarra and specifically its research center, CIMA, have gained a great deal of experience in rare hepatic diseases such as AIP. A research project funded by the European Union, AIPGENE (3), studied the potential of recombinant adeno associated virus-delivered human porphobilinogen deaminase (PBGD) gene into hepatocytes. Although preclinical and phase I clinical trials demonstrated the safety of this gene therapy, the efficacy clinical studies showed similar urinary accumulation of heme precursors before and after the administration of the treatment. This outcome encourage CIMA researchers to explore alternative therapies such as messenger RNA-delivered human PBGD (4) or recombinant PBGD proteins linked to apolipoproteins to target liver (4).

The work is framed within the rare disease acute intermittent porphyria (AIP) and it is quite unique. It does not represent a retrospective analysis of data generated in the past, on the contrary,

the models have been developed contemporary to the experimental studies contributing to improve experimental designs, data interpretability and making possible that exploratory conditions assessed *in silico* are taken into consideration in future clinical trials.

The computational model presented in *Chapter 1* represents an initial step towards modeling AIP and the first computational model to our knowledge that tackle this rare disease in a quantitatively way (5). Under the perspective that pharmacological effects are the result of drug action and progression of the disease, this work focuses on how the disease progresses once a perturbation scenario occurs. Model building was challenging due to the fact that observations were measured in urine of mice excreted every 24h, while the relevant disease processes were happening in the liver. Such data-related limitations highlights the advantage of integrating longitudinal data into a (semi-) mechanistic computational framework to extract the most from the gathered experimental information. This model was able to describe the excretion of the urinary biomarkers 5-aminolevulinic acid (ALA), porphobilinogen (PBG), and porphyrins before, during and after the induction of acute attacks by phenobarbital administration. It should be noticed that the measured entities represent the biomarkers measured in patients as well. To explore the model beyond the current control scenario, a theoretical exercise was performed by simulating the effects of the standard-of-care for AIP, hemin, to test the best time of administration before or during an acute attack. This model became the framework that future model-based analyses would utilize. Nevertheless, due to data constraints (the animal model did not receive any treatment), several mechanisms could not be included, and model simplifications were warranted.

The perturbation-disease model described in *Chapter 1* was expanded and improved in *Chapter 2* including data from treatment effects (6). Using the same framework, AIP was modeled by considering urinary data from not only porphyric mice, but also from wild-type mice, New Zealand rabbits and cynomolgus macaques. A new therapy (messenger RNA [mRNA] encoding human PBG deaminase [PBGD]) was tested on these animals. One important contribution from an experimental point of view was the longitudinal measurement of mRNA and the resulting PBGD activity in the liver. This additional piece of information allowed to characterize quantitatively and semi-mechanistically transcription and protein expression processes adding the pharmacodynamic contribution to the perturbation/disease and drug effect model. The use of data from different animal models and mRNA formulations resulted in the estimation of formulation- and animal-specific parameters, the latter providing the possibility to project drug effects in humans as a tool to select dose levels and formulation type. From a mechanistic point of view, several limitations were identified. For example, the model could not discriminate the dynamics between ALA and PBG, and it required of an extra inhibitory effect of PBGD effect on ALA synthesis.

Undoubtedly, the models described in *chapters 1* and *2* represent an innovative contribution, not exempted of drawbacks as the ones already accounted for in the discussion section. The question of how to overcome at least partly some of those limitations arises. The available information in literature to be leveraged into a dynamic "bottom-up" (i.e., systems pharmacology) type model for this particular disease is still scarce. Therefore, and under these circumstances, a look to the early efforts in pharmacokinetic (PK)/pharmacodynamic (PD) modeling might enlighten how to establish a development program to fill the data gap. The concept of experimental perturbation that forces the data to speak is key here. To illustrate this idea, the way that the development of tolerance and its impact on the heart rate effects elicited by nicotine was characterized, represents a very appealing example (7). By simply changing the time at which the second intravenous infusion starts with respect to the initial one, the generated PK and PD data allowed to build a semi-mechanistic and predictive model that described the tolerance effect caused by repeated administrations of nicotine.

The development program of new recombinant liver-targeted proteins contributed with appropriate data to improve the mechanistic structure of the so far perturbation-disease progression and drug effect model. The fact that the different formulations alter liver uptake of the therapeutics together with the differences across the liver and systemic circulation in the presence of the key enzymes of the heme pathway created a cocktail that allowed the incorporation of other elements in the model and identify additional mechanisms of the heme pathway. In particular, the role of heme controlling the synthesis of ALA by repressing the expression of ALA synthase (ALAS), and the effects of the porphyrinogenic drug phenobarbital on ALAS expression and heme reduction as the result of increasing the heme demand of cytochromes to account for the elimination of phenobarbital. As in the previous modeling exercises, simulations were performed. In this occasion, the impact of the different routes of administration, dosing schedules, and treatment start with respect to an eventual porphyric attack on the protective effects of the therapies was evaluated, providing additional means for selection of the best formulation and experimental setting for future clinical trials.

The analyses comprising this thesis were constrained to the pre-clinical arena since data in patients for any of the therapeutics described are still not publicly available at least. Nevertheless, a comparison with the drug givosiran is possible. Givosiran has been recently approved for the treatment of acute intermittent porphyria. As mentioned in the *Introduction* section, it is a small interference RNA (siRNA) linked to a trivalent N-acetylgalactosamine ligand that targets it to the liver. It inhibits the translation of the ALAS mRNA, resulting in a decrease in the accumulation of ALA and PBG (8). As it inhibits the first, rate-liming reaction of the heme biosynthesis pathway, the whole heme synthesis process is being turned off. This means that Givosiran could

have a potential side effect on the production of heme-dependent enzymes (such as cytochrome P450 enzymes), although this has not been proved yet (8).

The new therapy presented in this thesis, a recombinant PBGD (rPBGD) protein linked to the apolipoprotein AI (ApoAI), presents several advantages when compared to givosiran. First, its mechanism of action restores the impaired endogenous PBGD activity in liver, meaning that the heme biosynthesis pathway is not inhibited in any way. Second, the conjugation to ApoAI allows a successful drug targeting to liver. Finally, circulating recombinant PBGD molecules in blood also have a therapeutic effect by reducing the accumulation of systemic PBG levels due to the catalysis of the reaction from PBG to porphyrins, as shown in *Chapter 3*.

Similar PK (concentrations or PBGD activity values over time) and PD (longitudinal data of urinary or tissue heme precursor amounts) sampling designs were applied during the development of both therapies (givosiran and rPBGD). Nonetheless, the impact of model-based works to support their development has been different. On one hand, model development for Givosiran was limited and consisted on simple "top-down" approaches for PK and PKPD models (9). On the other hand, drug development of human and recombinant PBGD proteins have been fully supported by innovative computational models as shown in this thesis. This allowed not only to estimate PK and PD parameters, but to gain knowledge about AIP mechanisms and to extrapolate the results to other dosing regimens or even other species. We encourage experimental and clinical teams in the field of AIP to obtain longitudinal data in liver and/or blood after the administration of givosiran, rPBGD or any other current or new therapy, as it could greatly help to understand what molecular reactions and changes are happening during and after an acute porphyric attack, thus improving the already good prediction capability of computational models for AIP and leveraging more information for future clinical trials.

The mechanistic model framework provides answers not only to AIP, but to other porphyrias that are caused by mutations in the heme biosynthesis pathway such as ALA dehydratase porphyria (10), hereditary coproporphyria or variegate porphyria (11). These porphyrias also present accumulation of heme precursors and they are associated with acute attacks. The model could describe the dynamics of the heme precursors in liver (or in red blood cells) without any major change to the model structure, allowing to predict the accumulation of the biomarkers and the potential

In summary, this thesis shows how a mechanistic modeling framework for a rare disease is first designed, developed, and validated; and then is expanded and improved using new data and new conditions that can perturb the system enough to characterize hidden regulatory mechanisms. This framework can then be used to simulate hypothetical preclinical or clinical scenarios that inform the design of future preclinical experiments or even first-in-human phase I clinical trials.

Modeling in rare diseases is viable, but it needs the collaboration of clinical experts and pharmaceutical companies to advocate for longitudinal-based studies that can provide more informative data, thus better chances to build more helpful models.

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Conclusions

The main objective of this thesis was to build of a mechanistic modeling framework applied to acute intermittent porphyria (AIP), a rare disease, in order to characterize the disease dynamics and the effect of innovative therapies on restoring healthy levels of biomarkers in urine. The specific achievements have been described in the three chapters of this thesis and are summarized here as follows:

1. Biomarkers levels from the heme biosynthesis pathway measured every 24h in urine allowed the development of a semi-mechanistic disease model in AIP animals during and after a phenobarbital-induced acute porphyric attack.

2. The maximum phenobarbital concentration, the drug which induces the acute attack, caused a 300% increase in the synthesis of the biomarker ALA.

3. The response to new messenger RNA (mRNA)-based therapies was described by including in the previous disease model elements that represented mRNA kinetics in liver, porphobilinogen deaminase (PBGD) transcription and expression processes. A good characterization of the biomarker dynamics in urine for different animal species was achieved.

4. A PBGD activity increase of 8.9 pmol URO·mg protein−1·hr−1 was enough to prevent the 90% of phenobarbital-induced effects in AIP mice. Human extrapolation showed that the administration of 0.5 or 1 mg·kg−1 of SEQ-5 mRNA increased PBGD activity levels in healthy patients up to 37 or 56 days, respectively.

5. The computational approach that incorporated effect data obtained after the treatment with recombinant fusion PBGD proteins targeted to liver provided an adequate characterization of the pharmacological response as well as allowed to identify key processes in the heme biosynthesis pathway.

6. The *in silico* exploration of different therapeutic scenarios revealed a protective effect of up to 7 days on an acute porphyric attack after the subcutaneous administration of the fusion PBGD protein, while the intravenous administration was the optimal one once the acute attack has already started.

7. In summary, the integration of data from multiple sources, the previous information about the disease, therapies and the system; and the changes and assumptions established during model building resulted in a mechanistic computational framework which can provide numerous results that guide drug development or increase knowledge about the disease in other porphyrias or hepatic enzymopenias beyond AIP or the treatments displayed in this thesis.

Conclusiones

El objetivo principal de esta tesis doctoral fue la construcción de una estructura de modelado mecanicista aplicada a la Porfiria Aguda Intermitente (PAI), una enfermedad rara, para caracterizar la dinamia de la enfermedad y el efecto de terapias innovadoras en el restablecimiento de niveles normales de biomarcadores en orina. Los logros específicos han sido descritos en los tres capítulos de esta tesis y se resumen aquí de la siguiente manera:

1. Los niveles de biomarcadores de la cascada del hemo medidos cada 24h en orina permitieron el desarrollo de un modelo de enfermedad semi-mecanístico en animales PAI durante y tras un ataque agudo de porfiria inducido por el fármaco fenobarbital.

2. La máxima concentración de fenobarbital, inductor del ataque agudo, provocó que la síntesis del marcador ALA aumentara un 300%.

3. La respuesta a nuevos tratamientos de terapias basadas en ARN mensajero (ARNm) fue descrita incorporando en el modelo anterior de enfermedad elementos que representaban los procesos de cinética de ARNm hepático, trascripción y expresión de la enzima PBGD. Se obtuvo una buena descripción de las dinamias de biomarcadores en orina para distintas especies animales.

4. Un incremento de actividad de PBGD de 8.9 pmol URO·mg proteina^{-1}·hr^{-1} era suficiente para prevenir el 90% de los efectos generados por el fenobarbital en ratones porfíricos. La extrapolación a humanos demostró que la administración de 0.5 o 1 mg·kg^{-1} del ARNm SEQ-5 aumentaba los niveles de PBGD de pacientes sanos durante 37 o 56 días, respectivamente.

5. La aproximación computacional incorporando resultados de respuesta obtenida tras el tratamiento con proteínas de fusión de la enzima PBGD recombinante dirigidas al hígado permitió una adecuada caracterización de la respuesta farmacológica permitiendo a la vez identificar procesos relevantes en la cascada de señalización del hemo.

6. La exploración *in silico* de diferentes escenarios terapéuticos reveló un efecto protector de 7 días de duración sobre un ataque agudo de porfiria al administrar la proteína de fusión por vía subcutánea, mientras que la administración intravenosa era la más adecuada una vez comenzado el ataque agudo.

7. En resumen, la integración de datos de múltiples fuentes, la información previa de la enfermedad, tratamiento y sistema; y los ajustes e hipótesis planteadas durante la construcción de los modelos han dado lugar a un marco computacional mecanístico del cual se pueden extraer múltiples resultados que guíen el desarrollo de fármacos o que aumenten el conocimiento de la enfermedad en otras porfirias o enzimopenias hepáticas más allá de la PAI o de los tratamientos mostrados en esta tesis.

Appendix 1

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Research Article

Machine Learning Analysis of Individual Tumor Lesions in Four Metastatic Colorectal Cancer Clinical Studies: Linking Tumor Heterogeneity to Overall Survival

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Abstract. Total tumor size (TS) metrics used in TS models in oncology do not consider tumor heterogeneity, which could help to better predict drug efficacy. We analyzed individual target lesions (iTLs) of patients with metastatic colorectal carcinoma (mCRC) to determine differences in TS dynamics by using the ClassIfication Clustering of Individual Lesions (CICIL) methodology. Results from subgroup analyses comparing genetic mutations and TS metrics were assessed and applied to survival analyses. Data from four mCRC clinical studies were analyzed (1781 patients, 6369 iTLs). CICIL was used to assess differences in lesion TS dynamics within a tissue (intra-class) or across different tissues (inter-class). First, lesions were automatically classified based on their location. Cross-correlation coefficients (CCs) determined if each pair of lesions followed similar or opposite dynamics. Finally, CCs were grouped by using the K-means clustering method. Heterogeneity in tumor dynamics was lower in the intra-class analysis than in the inter-class analysis for patients receiving cetuximab. More tumor heterogeneity was found in KRAS mutated patients compared to KRAS wild-type (KRASwt) patients and when using sum of longest diameters *versus* sum of products of diameters. Tumor heterogeneity quantified as the median patient's CC was found to be a predictor of overall survival (OS) (HR = 1.44, 95% CI 1.08–1.92), especially in KRASwt patients. Intra- and inter-tumor tissue heterogeneities were assessed with CICIL. Derived metrics of heterogeneity were found to be a predictor of OS time. Considering differences between lesions' TS dynamics could improve oncology models in favor of a better prediction of OS.

KEY WORDS: cetuximab; individual tumor lesion dynamics; machine-learning; metastatic colorectal cancer; survival analysis; tumor size modeling.

INTRODUCTION

Model-Informed Drug Discovery and Development (MID3) (1) has demonstrated its usefulness to improve drug development in several cases, including the oncology area and the modeling of tumor size (TS) (2,3). TS is often expressed as the sum of longest diameters (SLD) of the individual tumor lesions (iTLs) measurable and defined as "target lesions" at baseline, as described by Response Evaluation Criteria in Solid Tumors (RECIST) (4). Each patient presents multiple iTLs, which can be primary or metastatic and located in several organs or tissues. This means that all tumor lesions, regardless of their location and status, are reduced to this single SLD value, also called the total TS, at each assessment visit within a patient. The iTLs are assessed throughout the clinical study; the SLD is derived at each time point and then categorized to quantify the tumor response to the treatment (4). Observed or model-derived TS metrics, such as the early tumor shrinkage (ETS, relative reduction of total TS at certain time points) or time to tumor growth, have been shown to be predictors of overall survival (OS) (5).

As an oversimplifying measure of cancer progression, the use of total TS may cause a loss of information with respect to tumor heterogeneity that could carry valuable information to

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Tumor Size Quantification

Lesions TS was quantified either by computed tomography scan or magnetic resonance imaging. At baseline, iTLs were defined as measurable lesions representative of all involved organs, with a maximum of 5 lesions per organ and 10 lesions in total. According to studies protocols, the same method of assessment and the same imaging technique was used to characterize each identified and reported lesion at baseline and at each subsequent imaging time point. In the CRYSTAL, 045, and OPUS studies, iTLs were bidimensionally evaluated by using the modified WHO criteria (20,21), which quantifies the total TS by measuring the longest and perpendicular diameters of iTLs and then, deriving the so-called SOPD, sums of the products of diameters. In APEC, the assessment of response was performed according to RECIST (4,22) which uses the sum of longest diameters SLD of iTLs as a measure of total TS. Thus, unidimensional measurements were collected for iTLs in APEC, while bidimensional measurements were available for the other studies (23). In addition to the recorded TS measures over time, information about the lesion site was collected for all iTLs as text in the case report form for all studies. The lesion type was also coded as follows: primary, metastatic, or node. Calculated SLD or SOPD of lesions selected as target lesions and the recorded information on non-target lesions and new lesions were used to derive response and progression outcomes throughout the studies.

Dataset Preprocessing

iTL data from the four clinical studies was extracted from the clinical database. Patients with only the tumor assessment at baseline as well as tumor data measured after tumor surgery procedures were excluded from the analysis. The main CICIL analyses presented in this work used the longest diameter as a single TS metric being available across all studies. In the three studies having bidimensional measures of TS available for each iTL, CICIL was re-run for comparisons of results with the two metrics.

CICIL Methodology

The previously developed CICIL methodology, implemented in a Java-based platform, was used to evaluate the similarities or differences between iTL dynamics (inter-organs or intra-organ) (11). This methodology consists of 3 steps: (i) iTLs are classified based on their location and type described by expert physicians, (ii) cross-correlation (CC) values are estimated to assess the degree of similarity between dynamics of two lesions, and (iii) similar cross-correlation values are automatically grouped into clusters using the K-means clustering method (24). This approach can be easily applied to either the bi-dimensional product (WHO criteria), the longest diameter (RECIST criteria) or any future emergent volumetric measurement provided by progresses in tumor imaging and/or tumor size collection. Thus, we could analyze lesion sizes regardless of tumor evaluation criteria for diagnosis of progression of disease adopted in the studies.

Two kinds of CICIL analyses were performed: inter-class analysis and intra-class analysis. On one hand, the inter-class analysis uses the sum of lesions TS within each patient's individual organs defined in the CICIL classification. They are called class-related target lesions (cTLs). Therefore, each patient shows a single cTL for each organ/tissue. Then, the CC value of the pairs of cTLs the patient presents with is computed to assess the difference in lesion dynamics between organs or tissues. This analysis then includes only patients presenting iTLs in more than one tissue. On the other hand, the intra-class analysis uses the iTLs from a single organ or tissue to compute the CC values for the pairs of iTLs in that organ or tissue. This is performed to determine the differences in lesion dynamics between iTLs within the same patient's individual organ or tissue. Only patients presenting more than one iTL within the same organ are included in the analysis of that specific class. Figure 1 shows an overview of the CICIL methodology.

Step 1: Rule-Based Classification

iTLs were classified according to the same methods described in (11), which were defined for two other mCRC clinical studies. Several keywords were defined for each class in the classification text file of CICIL. These were based on the recorded tumor location and anatomical and physiological features observed on tumor lesions of these organs. The CICIL platform performed the automated classification process by recognizing the defined keywords and locations of each lesion in the extracted clinical dataset. Before running the classifier tool, missing or wrong organ information was checked (and corrected, if needed) during dataset preprocessing. Lesions with missing organ information were classified in the general "Unclassified lesions" class.

Step 2: Cross-correlation Analysis

In order to automatically compare hundreds of TS dynamics, while keeping information on the lesion dynamics across the whole study, the non-parametric comparison of two TS time courses, treated as time series, was performed by estimating the CC (25). The CC values can range from −1 to 1. CC values equal or close to 1 indicate similar tumor dynamics for the two compared lesions. CC values equal or close to −1 indicate opposite profiles. In contrast, CC values close to 0 indicate undefined relationships (i.e., not distinguishable) or small trends towards similarity or difference depending on the sign and absolute value of the CC. Thus, the CC value obtained from two TS dynamics was used as a metric of similarity or difference between such lesion dynamics.

CC values were calculated without accounting for any delay between TS dynamics (zero-time shift) as well as at shifting one of the lesions over the other (and vice versa) in time to maximize their CC value. The number of assessments per lesion allowed us to check up to 12 time shifts in both directions. Details on CC calculations are reported in (11).

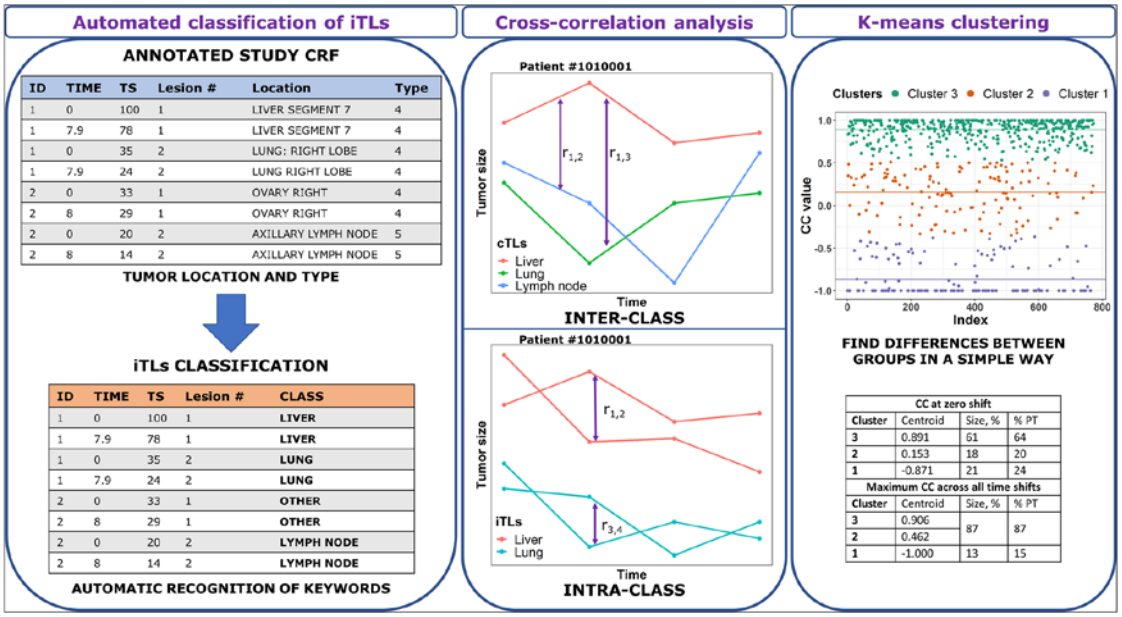


Fig. 1. CICIL methodology overview. Clinical trials present in their case-report forms (CRFs) information about tumor size, time, tumor location, and tumor type of individual tumor lesions (iTLs). This data can be used to classify these iTLs. Then, the degree of similarity between the time-course of those lesions in a patient can be computed with the cross-correlation, both between lesions belonging to different classes (cTLs, inter-class) and between iTLs within a class (intra-class). Similar cross-correlation coefficients (CCs) are grouped by applying the k-means clustering technique to find differences between groups of CCs

Step 3: K-Means Clustering

K-means clustering is a ML unsupervised clustering technique (24). As the last step of CICIL, this method was used to group the different CC values into clusters. Then, differences between these groups or clusters could be assessed based on the centroid value (the average CC value of each cluster) and the percentage of lesion comparisons (i.e., CCs) and patients in each cluster. Two arbitrary cutoffs were established for easy interpretation of results: clusters with centroids below − 0.35 were considered as an indication of different lesion dynamics, whereas those with centroids above 0.35 were considered as pointing to similar dynamics. Clusters with values in between were considered as suggesting undefined relationships.

Beforehand, the number of clusters was selected with the elbow method which allows to assess and then choose the smaller number of clusters having a lower sum of squared errors (SSE) (26). If SSE *versus* the number of clusters were plotted, the arm would be the plotted line and the elbow would correspond to the optimal number of clusters.

Survival Analysis

Overall survival time data were extracted from the four studies in order to perform survival analyses and to assess whether results from the CICIL analyses could be used as predictors of survival time. Kaplan-Meier plots were obtained, log-rank tests were computed, and a Cox proportional hazards model was fitted to the data. One of the tested predictors was the median CC for the inter-class analysis, which was computed for each cetuximab arm patient as the median of CC values at zero-time shifts obtained from the different lesion pairs (e.g., liver-lung or liver-node) within a patient. Given its relationship with patients' response, the KRAS status was also tested as a predictor.

Software

Dataset preprocessing was carried out in R version 3.5 (27) by importing the clinical SAS® dataset with the function *read_sas* from the package *haven* 2.2.0 (28). The CICIL methodology was performed in its Java-based software version 1.0.4. (11). For the survival analysis, R was used to run statistical tests and models, along with the *survival* package (29).

RESULTS

For the CICIL analysis, 1781 patients from the four previously described clinical studies were included: 1127 patients from CRYSTAL, 271 patients from APEC, 61 patients from Study 45 and 322 patients from OPUS. Separate CICIL analyses were performed for each study by considering different subsets of data: (i) all patients, (ii) patients receiving cetuximab, and (iii) KRASwt patients receiving cetuximab. All patients from the APEC and Study 045 studies received cetuximab. Figure 2 shows a tree diagram with the number of patients considered at each stage of this work.

Appendix 1

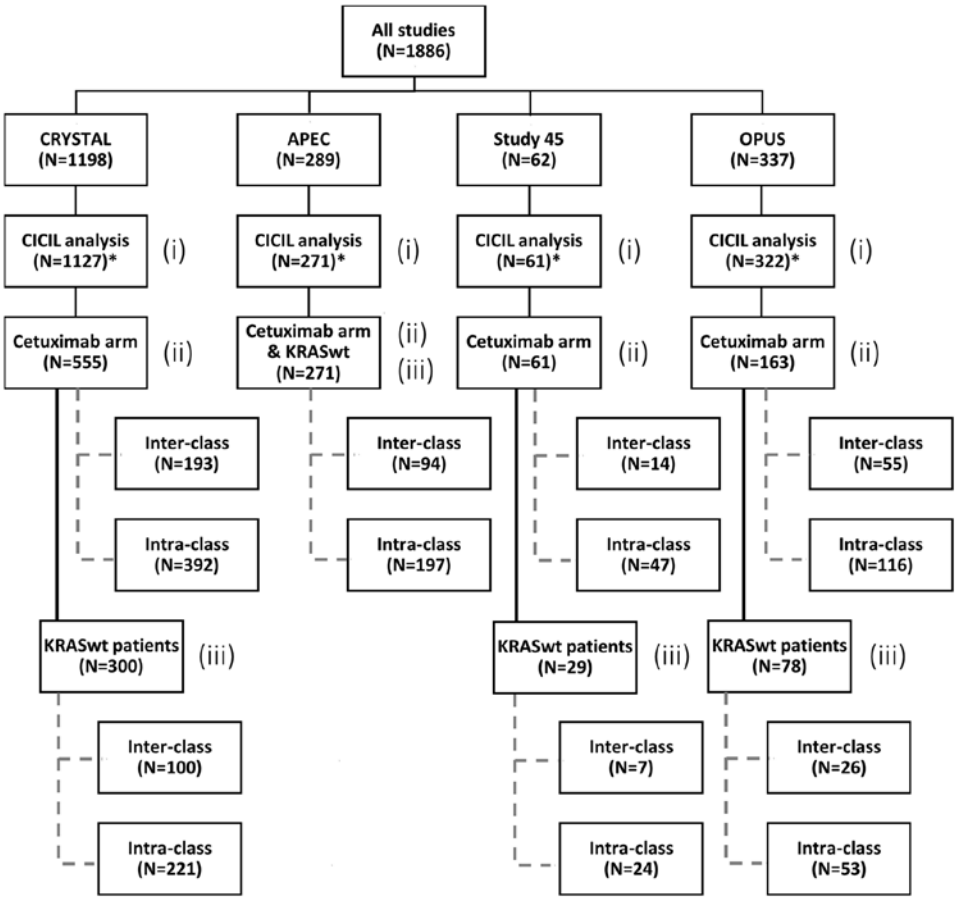


Fig. 2. Tree diagram showing the distribution of patients across different subsets of CICIL analyses. Analyses on different subsets of patients were performed: (i) all patients, (ii) all patients receiving cetuximab either as monotherapy or in combination (cetuximab arm), and (iii) KRASwt patients receiving cetuximab. All APEC patients were KRASwt. *Not all patients were able to enter the CICIL analysis. Those without more than one tumor size (TS) assessment or with missing organ information were excluded

Classification

iTLs were classified into eight classes based on the organ keywords and location information. This classification was adapted from the previous work by Terranova *et al.* (11) and found to be suitable for our clinical studies. The defined and adapted classes were as follows: "Liver", "Lung", "Lymph node", "Other respiratory organs", "Other digestive organs", "Other specified organs", "Primary lesions", and "Unclassified lesions". The "Primary lesions" class was populated with non-metastatic lesions, whereas "Unclassified lesions" class contained lesions without proper organ information.

Similarly to the previously described CICIL work (11), a second classification step was performed, as the "Other respiratory organs", "Other digestive organs", "Other specified organs", "Primary lesions" and "Unclassified lesions" classes presented less than 30 patients. These classes with low numbers of patients were combined into the "Other" class. Table II shows the total number of cTLs (equal to the number of subjects with at least one lesion in that class) and the number of iTLs for each subset of data (all patients, patients who received cetuximab, and KRASwt patients who received cetuximab) across classes. The liver class represented 68% of all the iTLs measured across all studies. Lung and lymph node classes accounted for 12% for iTLs each one. The class Other only included 8% of iTLs.

Inter-class Analysis

All Patients

Only patients with more than one class could be included in the inter-class analysis, so this reduced the number of available cTLs for the analysis to 926 cTLs (from 404 patients) in the CRYSTAL study, 215 cTLs (from 94 patients) in the APEC study, 30 cTLs (from 14 patients) in Study 045, and 220 cTLs (from 99 patients) in the OPUS study.

When data from the four studies were analyzed together, three clusters were used. About 61% of lesion pairs (from 64% of patients), grouped into cluster 3, showed similar lesion dynamics. Cluster 2 contained 18% of comparisons. This cluster presented a small positive correlation between

Table II. Classification of Individual Target Lesions

Class name	CRYSTAL			APEC	Study 45		OPUS			All studies		
	ALL	Cetuximab arm	KRASwt	ALL	ALL	KRASwt	ALL	Cetuximab arm	KRASwt	ALL	Cetuximab arm	KRASwt
Liver	884 (2867)	434 (1420)	227 (745)	176 (525)	51 (153)	26 (89)	265 (774)	139 (421)	63 (197)	1376 (4319)	800 (2519)	487 (1546)
Lung	252 (521)	132 (262)	63 (113)	68 (155)	10 (22)	5 (7)	49 (85)	24 (43)	8 (12)	379 (783)	234 (482)	141 (284)
Lymph node	261 (488)	110 (193)	62 (110)	72 (141)	4 (7)	0	60 (113)	33 (63)	19 (37)	397 (749)	219 (404)	148 (278)
Other	250 (319)	121 (151)	73 (89)	74 (102)	12 (12)	6 (6)	70 (85)	36 (42)	21 (25)	406 (518)	243 (307)	170 (218)
Total	1647 (4195)	797 (2026)	425 (1057)	390 (923)	77 (194)	37 (102)	444 (1057)	232 (569)	111 (271)	2540 (6369)	1496 (3712)	946 (2326)

The number of class-related target lesions (cTLs, sum of lesions of a class for a patient) is presented in the table. Individual target lesions (iTLs, 1 or more than 1 per patient) are shown in parenthesis. ALL, target lesions from all patients included in the CICIL analysis. Cetuximab arm, patients who received cetuximab either as a monotherapy or in combination with chemotherapy: FOLFIRI (folinic acid + 5-fluorouracil + irinotecan) or FOLFOX (folinic acid + 5-fluorouracil + oxaliplatin). KRASwt, target lesions from KRASwt patients only. All patients in APEC and Study 45 studies received cetuximab; thus, the "Cetuximab arm" columns are omitted. APEC only included KRASwt patients: in this case only the "ALL" column is shown

lesions. Remaining comparisons were included into cluster 1, with a centroid close to −1 indicating that those lesions presented different dynamics. When time shifts were taken into account, the percentages of lesion comparisons and patients within clusters with similar dynamics or positive correlation (clusters 2 and 3) increased to 87%. Thus, less tumor heterogeneity across classes was suggested when taking into account time shifts of lesions dynamics.

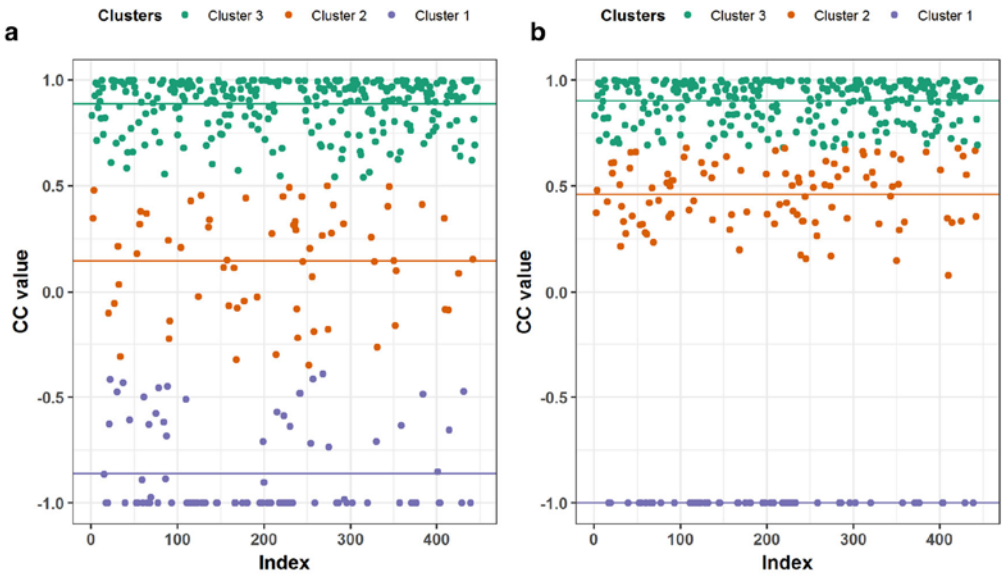
Comparison: Cetuximab Arm Patients Versus Non-cetuximab Arm Patients

Patients in the cetuximab arm were used to look for potential differences from patients not receiving cetuximab. The optimal number of clusters was 3. Larger percentages of lesion pairs (65%) and patients (68%) in the cetuximab arm were found in cluster 3 compared to the group of patients not receiving cetuximab (56% lesion pairs from 58% of patients). Nevertheless, the percentage of lesion pairs (21%) and patients (23%) in cluster 1 for the cetuximab group was similar to those in the group not receiving this therapy (21% lesion pairs from 25% of patients, respectively). This points to a similar tumor heterogeneity in lesion dynamics between the two subgroups. Results from the CICIL analysis on patients receiving cetuximab are shown in Fig. 3. Inspection of distributions of time shifts at which the maximum CC values were achieved within cluster 3 suggests good synchronicity (59% of maximum CCs at zero-time shift) between similar cTLs dynamics. Cluster 2 showed that small shifts (from −1 to +1) accounted for most of the maximum CC values in this cluster. Finally, cluster 1 presented all maximum CC values at zero-time shift. Indeed, these came from lesion pairs with only two tumor assessments, thus not allowing any series shift in time. Overall, 23% of patients in the cetuximab arm presented different lesion dynamics at zero-time shift, but if time shifts are considered (especially small lags like ±1, as stated above), only 14% of patients showed opposite lesion dynamics. An illustrative representation of the impact of small time shifts on CC is provided for cTLs with different CC values at zero-time shifts in Supplementary Figure S1.

Comparison: KRAS Wild-Type Patients Versus KRAS Mutated Patients

The CICIL analysis was also performed on KRAS subsets of patients to compare KRASwt *versus* KRAS mutated (KRASmut) lesion dynamics. Results based on the combined dataset of the four clinical studies showed that more similar lesion dynamics were found in patients with KRASwt mCRC lesions (72% lesion pairs belonging to 74% of patients) than in patients with KRASmut lesions (52% lesion pairs belonging to 57% of patients). Cluster 1 (different lesion dynamics) included 16% lesion pairs from 18% of patients in the KRASwt group and 29% of lesion pairs from 31% of patients in KRAS mutated group, respectively.

The same pattern was observed when the analysis was performed on the CRYSTAL and OPUS studies separately. No conclusions can be drawn for APEC and Study 045 studies: APEC had no KRASmut patients and Study 045 had



c

CCs measured at zero-time shift				Maximum CC values			
Cluster	Centroid	Size, %	No. patients, %	Cluster	Centroid	Size, %	No. patients, %
3	0.89	65	68	3	0.90	87	87
2	0.15	15	17	2	0.46		
1	-0.86	21	23	1	-1.00	13	14

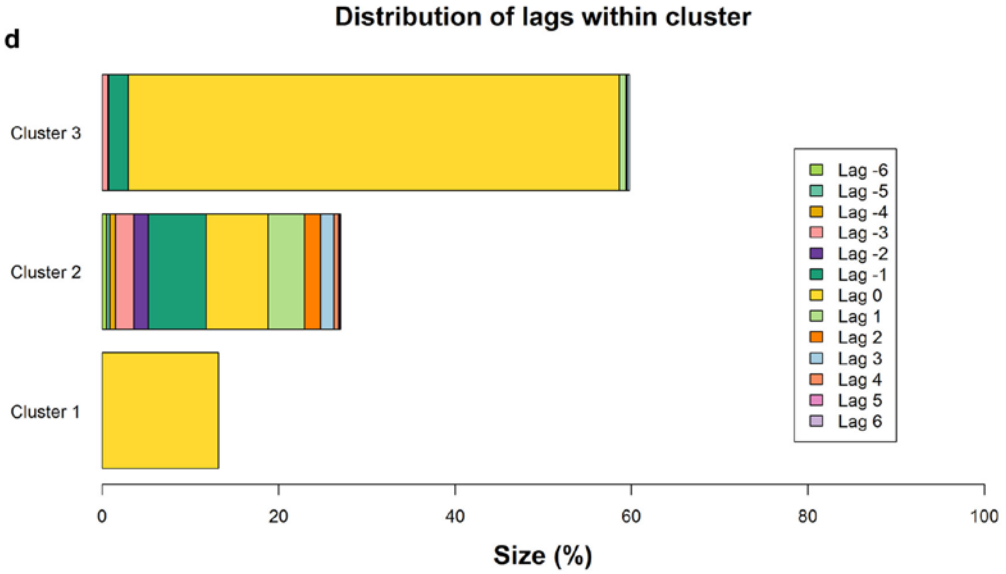


Fig. 3. Inter-class analysis results for the combined data from the four clinical studies for those patients who received cetuximab, either as a monotherapy or in combination with the FOLFIRI (folinic acid + 5-fluorouracil + irinotecan) or FOLFOX (folinic acid + 5-fluorouracil + oxaliplatin) regimens. Results at zero-time shift (**a**) and when maximum cross-correlation coefficients (CC) values are achieved (**b**) are shown. Horizontal lines show the centroids for each cluster. Table in **c** details the results of this inter-class analysis for patients who were administered cetuximab. If the cluster centroid value was above 0.35, CCs from that cluster were considered to show similar dynamics and summed to CCs from cluster 3. Note that patient percentages accounted for more than 100%, because a single patient can have lesion pairs in different clusters. **d** The distribution of time shifts or lags at which the maximum CCs were obtained, within each cluster

a low number of eligible patients (14). See supplementary Table SI for more details.

Comparison: TS Measured as Product of Diameters Versus TS Measured as Longest Diameter

For the three studies with bidimensional TS measurements available, CICIL was re-run by using the SOPD of cTLs. Results were then compared to those previously obtained with SLD.

When all studies with SOPD measurements were analyzed together, similar percentages of lesion pairs (65% and 63% with SLD and SOPD, respectively) and patients (67% in both groups) were found in cluster 3. Percentages of lesion pairs (21%) and patients (23%) in cluster 1 were the same for both analyses. Nevertheless, the CRYSTAL study, the largest study in this work, presented larger percentages in cluster 3 with SOPD than with SLD measurements, both with respect to lesion pairs (60% *vs* 56%, respectively) and to patients (66% *vs* 62%, respectively). Cluster 1 included 22% *versus* 25% of lesion pairs and 23% *versus* 26% of patients, with SOPD and SLD, respectively.

Study 045 results displayed the same trend as CRYSTAL. However, due to the low number of lesion pairs, this result might not be as informative as the ones obtained in other studies. For the OPUS study, the percentages of lesion pairs and patients in clusters 2 and 3 were higher for SOPD than for SLD, but the same percentages were found in cluster 1 for both metrics. Overall, no major differences between SLD and SOPD results were shown for this study. See Supplementary Table SII for more details.

Intra-Class Analysis

The CICIL methodology was also applied to assess the differences across iTL dynamics grouped into the same class. Therefore, CCs were computed across iTLs from the same class within the same patient. Supplementary Table SIII shows the intra-class analysis results for each class for the combined dataset of patients treated with cetuximab.

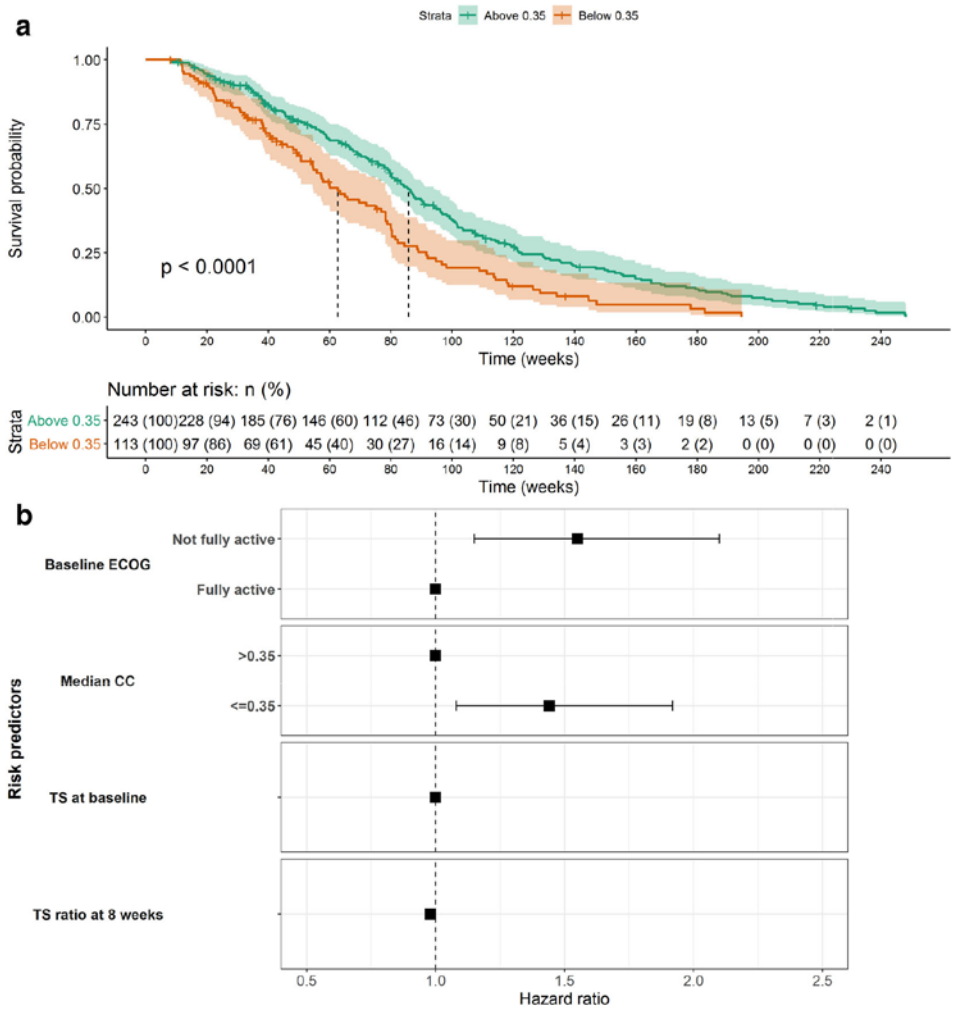
Overall, cluster 3 or both clusters 3 and 2 (provided the latter one presented a high-value centroid, i.e., greater than 0.35), included 71–88% of CC values for all the considered classes ("Liver", "Lung", "Lymph node", and "Other"). This means most lesion pairs showed similar lesion dynamics within every class. When time shifts were considered, the three main classes ("Liver", "Lung", and "Lymph node") decreased their percentage of CCs in cluster 1 (different lesion dynamics) from 8–23% at zero-time shift to 4–14% at maximum CC values. The "Other" class also decreased its percentage of CCs in cluster 1 from 29% at zero-time shift to 14% at maximum CCs.

Application of CICIL Results into Survival Analysis

Following the inter- and intra-class analyses, we assessed whether a metric related to the CC values (representative of tumor heterogeneity) could be predictive of OS. The inter-class median CC for each patient was derived as a unique metric to then assign the patients to two groups: patients with a median CC value equal to or below 0.35 and patients with median CC value above 0.35. The CC value of 0.35 was selected because it was considered an appropriate conservative threshold. Setting a higher threshold would have meant that more CCs would have been considered to present different dynamics. Nevertheless, similar results were obtained when considering a threshold of 0.5.

A log-rank test was first performed on the meta-analysis pooled dataset, and the median CC value was found statistically significant (p value $= 3.42 \times 10^{-5}$). When each study was assessed individually and for any subset of data, only CRYSTAL showed statistically significant results (p value equal to 7.15×10^{-5}). A Kaplan-Meier plot for the meta-analysis pooled data was then used to estimate median survival time in each group, which was 85.9 weeks (95% confidence interval (CI) 79.7–94 weeks) for those patients whose CC was above 0.35 and 62.7 weeks (95% CI 54.6–78.3 weeks) for the other group of patients. Figure 4a shows the Kaplan-Meier plot for the meta-analysis, stratified by the two CC groups. The potential confounding between KRAS status and heterogeneity quantified as median CC was also evaluated by deriving Kaplan-Meier plots stratified by the median CC (categorized as before) for KRASwt and KRASmut patients separately. In both groups, the median CC was found to be significant (p values equal to 0.0024 and 0.017 for KRASwt and KRASmut groups, respectively).

A multivariate Cox proportional hazards model was fitted to the meta-analysis pooled dataset using several risk predictor variables: KRAS status (categorized as KRASwt or KRASmut), ECOG performance score at baseline (as fully or not fully active), TS at baseline (continuous variable), and the ETS defined as the TS ratio at 8 weeks as a continuous variable ($TS_{ratio} = \frac{TS0-TS8}{TS0}$, where TS0 is the TS at baseline and TS8 is the TS at 8 weeks). These predictors had been already selected in a previous internal analysis based on total TS, and they were found to be statistically significant. The patient median CC value was added on top of this multivariate Cox model, both as a continuous and as a binary variable, to test its statistical significance as a predictor of risk. Results for the model containing the continuous median CC variable showed statistical significance for all predictors, except for the KRAS status which was significant in the univariate analysis but dropped from the final models (p value > 0.05). The p value of the median CC was 0.006 with hazard ratio (HR) of 0.74 (95% CI 0.60–0.92). This indicates that a CC value increase of one unit (e.g., from -0.5 to 0.5, thus reducing tumor heterogeneity) would lead to a decrease in the risk of death of 26%. Although the KRAS status itself was not significant, the addition of an interaction term between the KRAS status and the continuous median CC resulted to be significant (p value $= 0.022$) and suggested that the effect of our heterogeneity measure was less pronounced in KRASmut patients with an average increase of the risk in this patient population by 60%. If median CC was introduced into the multivariate Cox model as a categorized binary variable reflecting the two groups (median CC above or equal to/below 0.35), the same predictors were significant. The median CC presented a HR of 1.44 (95% CI 1.08–1.92, p value $= 0.012$) for the group equal to or below 0.35 (i.e., more tumor heterogeneity). These results are shown in Fig. 4b and c. This means that patients with the median CC



c

Predictor	Categories	Patients	HR	95 % CI HR	P-value
ECOG at baseline	Fully active	77	1 (ref.)	-	-
	Not fully active	231	1.55	1.15-2.10	0.004
Median CC	Above 0.35	214	1 (ref.)	-	-
	Below 0.35	94	1.44	1.08-1.92	0.012
TS at baseline	Continuous variable	308	1	1.00-1.00	<0.001
TS ratio at 8 weeks	Continuous variable	308	0.98	0.98-0.99	<0.001

Fig. 4. Survival analysis for the cetuximab arm of the meta-analysis pooled dataset. The Kaplan-Meier plot stratified by the median cross-correlation coefficient (CC) value is shown in (**a**). The median CC used as a predictor was categorized into two groups: median CC equal to or above (green) and below (orange) 0.35. The dashed lines show the median survival time for each group. The *p* value from the log-rank test is shown in the plot. Below the Kaplan-Meier plot, the number of patients (with percentages in parenthesis) at risk of death at each time is shown in a tabular format. The survival forest plot obtained from the final multivariate Cox proportional hazards model is shown in (**b**). ECOG score at baseline, tumor size (TS) at baseline, TS ratio at 8 weeks, and median CC predictors were assessed as predictors. Results are shown in **c** reporting the number of patients, hazard ratios (HR) along with their 95% confidence interval (95% CI), and *p* values (statistical significance) for each predictor included in the multivariate Cox proportional hazards model

equal to or below 0.35 presented an average 44% increase in the risk of death. Of note, the lack of significance of KRAS status in the multivariate analysis was true only on this reduced CICIL analysis set which included only subjects having the median CC available (i.e., iTLs in more than one class). Indeed, *p* values smaller than 0.001 were

obtained when running the analysis on all patients (including those without the median CC).

DISCUSSION

Despite enriching data analysis in oncology, lesion heterogeneity has been often ignored or disregarded in the TS modeling, as also highlighted by the limited number of published works in this field (9,10). This is due to the complex models and methods needed to consider differences between organs or tissues, as well as the intra-tumor heterogeneity within an anatomic area. Models with such features are complex and computationally expensive. Thus, being able to efficiently assess and quantify the heterogeneity in iTLs TS dynamics prior to any modeling analysis is extremely valuable to inform and to guide the best modeling strategies. The recently proposed CICIL methodology addressed this objective by exploiting ML techniques to inform subsequent modeling steps (11). This approach provides a user-friendly and automated framework to quantify heterogeneity in tumor dynamics at different levels: (i) between iTLs within an organ and (ii) between lesions located in different tissues. Results can be used to make data-driven modeling decisions, for example, about the use of total TS *versus* iTLs TS, and about the level (within or across tissues) of tumor heterogeneity to be accounted for in the model.

In this work, we used CICIL to analyze TS data of patients' iTLs from four mCRC studies investigating cetuximab treatment. The classification of iTLs considered the three main lesion classes "Liver", "Lung", "Lymph node", and the general class "Other". The low number of iTLs for the least frequent classes (i.e., "Other respiratory organs", "Other digestive organs", "Other specified organs", "Primary lesions" and "Unclassified lesions") did not allow us to assess intra-tumor heterogeneity in those classes separately and they had to be pooled into a single class called "Other". Results indicated that the majority of iTLs were located in the liver (68%). Liver was also the most representative class in a previous analysis of two other cetuximab mCRC clinical studies (11). This is in line with available literature highlighting the liver as the most common site of metastasis in mCRC patients due to its anatomical position with respect to the portal circulation (30,31).

CICIL results from the intra-class analysis indicated little intra-tumor heterogeneity in TS dynamics of patients' iTLs from the same classified tissue (less than 23% of iTL pairs at zero-time shift across the three main classes). Such results were obtained with a rich dataset including 863 patients and 2990 iTLs across studies which supports the robustness of the conclusions. Thus, we considered it conceivable to neglect intra-tumor heterogeneity in subsequent subgroup analyses performed in this work.

In the inter-class analysis, tumor heterogeneity between lesions located in different tissues (cTLs) was found to be higher. In particular, in the analysis based on the cetuximab arm, 36% of CCs or cTLs pairs (from 38% of patients) were found to follow different TS dynamics at zero-time shifts. This percentage is also marginally larger than the one (35% lesion pairs from 30 to 35% patients) obtained in a previous work (11), and it is mainly driven by the CRYSTAL study (44% lesion pairs from 45% patients) as the largest study in this work. When time shifts were considered, such percentage of CCs dropped to 13%. This points to the possibility that time delays could account for some of the observed tumor heterogeneity in the patient, e.g., a delayed response to the drug in some tumor tissues. It should be noted that in both analyses some patients presented iTLs with only two TS assessments, which made their CCs not as informative as those coming from lesions with more than two TS measurements.

Cetuximab patients with cTLs expressing KRASwt genetics showed less tumor heterogeneity in TS dynamics than KRASmut patients. Cetuximab, as an EGFR inhibitor monoclonal antibody, presents lower efficacy if lesions present with KRAS mutations (32). Therefore, we may relate this increased tumor heterogeneity in KRASmut patients to their reduced response to Cetuximab.

For those studies that measured the longest diameter and the perpendicular one, inter-class results obtained with the longest diameter were compared to those using the product of diameters as TS metric. The combined analysis from the pooled dataset did not suggest any differences between results obtained with SOPD and SLD metrics. Nevertheless, results for the CRYSTAL study showed smaller differences in cTL dynamics and then in tumor heterogeneity when SOPD was used as the TS metric (38% with SOPD *vs* 45% with SLD). This may point to a better characterization of tumor heterogeneity when bidimensional data are collected for this case study. Indeed, literature shows that such differences between TS metrics depend on the kind of cancer: in some cases, there are no differences between unidimensional or bidimensional measurements (33), while in other cases, there are significant differences between results obtained with these two metrics (34,35).

Tumor heterogeneity results obtained in the inter-class analysis were found to be a predictor variable of OS time in the form of median CC. Its significance was proven both alone (log-rank test and the Kaplan-Meier plot) and in combination with other known risk predictors (multivariate Cox proportional hazards model). Increased risk of death with increased heterogeneity was suggested by all tested models. Interestingly, the model including a significant interaction term between the heterogeneity metric and the KRAS status suggested a relevant decrease of risk (about 40%) with decreased heterogeneity in KRASwt patients, but a small decrease (about 3%) in KRASmut patients. This points to a reduced impact of heterogeneity in TS dynamics on risk for KRASmut patients which is however already associated with a lower treatment effect in this subpopulation. Pharmacokinetic-tumor size-overall survival relationships can be affected by immortal time and selection bias (36), in particular, in situations without dose-ranging data. As the estimation of our tumor heterogeneity measure is related to the number of tumor assessments the patient had over the study and this depends on the immortal time, the potential for immortal bias cannot be excluded. More sophisticated models including time-dependent covariates allowing a change in status over time may be tested in future works to overcome the potential for immortal time bias.

As one of the major outcomes of this work, such results further highlight the impact of tumor heterogeneity on tumor response and the importance of including it into survival

analyses. Besides, modeling in the oncology arena could benefit from including measures of tumor heterogeneity data such as CC values. For example, the use of "tissue-agnostic" datasets in which individual lesions are considered and grouped based on the degree of similarity in their TS dynamics could improve the performance of TS models in favor of a better prediction of OS time.

CONCLUSIONS

The CICIL outcome obtained from a large dataset of tumor measures was assessed with respect to different factors (genetic mutations, tumor metrics), and its direct link with a clinical endpoint was quantified. Comparisons between KRASwt and KRASmut patients indicated less heterogeneity in tumor lesions dynamics in the KRASwt subgroup which is known to well respond to Cetuximab treatment. The method used to measure the lesion TS did not lead to different results except for the single-study analysis of CRYSTAL where an apparent heterogeneity was compensated and reduced when including the perpendicular diameter and obtaining the SOPD. An increased risk of death with increased heterogeneity was suggested by all tested models, especially in KRASwt patients. A reduced impact of heterogeneity in TS dynamics on risk for KRASmut patients was indicated.

The identification of a new metric of tumor heterogeneity related to a clinical outcome as OS is a relevant finding in the exploration of TS metrics that can inform clinical development decisions. This further supports the use of continuous TS response metrics as endpoints in early clinical oncology studies in order to improve design efficiency.

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Appendix 2

Relevance of primary lesion location, tumor heterogeneity and genetic mutation demonstrated through tumor growth inhibition and overall survival modeling in metastatic colorectal cancer

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