Gut feelings

Chapter 7

Summary and General discussion

The intestinal tract is a vital organ due to its numerous functions. It is not only involved in digestion, metabolism, and absorption of nutrients and drugs, but also in immune, endocrine, and neuromotor responses [1, 2]. Therefore, it is of great importance to maintain the intestines healthy and functional. In fact, intestinal damage during drug treatments, especially anti-cancer ones, is regarded as a serious adverse effect that may require dose reduction to prevent further damage [3]. Hence, intestinal damage can be dose-limiting and lead to dosing adjustment or interruption of the therapy. Nevertheless, intestinal toxicity caused by drugs has been underestimated thus far and a very limited number of studies have focused on this aspect of pharmacotherapy. Moreover, compared to other main organs such as the liver, heart, or kidneys, there has been less progress in investigating intestinal toxicity, thus available data is very limited. Therefore, one goal of our studies was to generate new data on intestinal toxicity induced by different chemotherapeutics. Transcriptomic responses were aligned with cytotoxicity endpoints, proteomics, and metabolomics in order to improve our understanding of the perturbed biological processes in intestinal cells and, ultimately, support the future establishment of intestinal toxicity predictive models for drug safety assessment.

Up until recently, the *in vitro* investigation of drug-induced intestinal toxicity has been conducted in conventional 2-dimensional (2D) cultures, which are mainly originated from colon cancer cells, such as the frequently used Caco-2 or HT-29 cell lines [4]. The use of these cancer-derived cells is not the most ideal approach to gain insight into drug-induced gene expression changes in healthy human tissue, as cancer cells often modulate their genetic traits to increase proliferation and resistance to drugs [5, 6]. As a result, this makes the already challenging translatability of *in vitro* to human conditions even more difficult. Although animal models take pharmacokinetic effects into account that cannot be studied *in vitro*, animal research is expensive and it does not accurately reflect drug responses in humans either. This is evidenced by attrition rates in clinical trials demonstrating that rodent-derived data poorly translate into human risks, being only 71% concordant [2, 7, 8]. Consequently, animal research often leads to failure of drug safety assessment during clinical testing phases, which delays drugs´ approval [8]. On the other hand, clinical studies performed so far are also limited in the sense that they usually include colon cancer samples from patients but do not include the healthy section of the exposed tissue. In addition, studies include therapies with a combination of drugs rather than monotherapy with the target drug. These complexities contribute to the limited investigation of transcriptomic signatures underlying drug-induced toxicity in healthy intestinal tissue.

In an attempt to overcome the fact that traditional 2D *in vitro* cultures and rodent models poorly reflect most human risks during clinical tests, a more advanced culture model was adopted in this work, namely three-dimensional (3D) culture models of human intestinal organoids. Sato et al. (2009) was the first investigator who developed long-term intestinal organoids from stem cells of the small intestine crypts, being the first major

technological advance in 3D culture [9]. Due to this remarkable work, it is now possible to perform long-term culture of organotypic 3D systems of any human tissue or organ [10-15]. Organoids may present features quite similar to *in vivo* tissue regarding proliferation, differentiation, and behaviour [16]. Therefore, they are regarded as the future tool to study biological processes from cellular differentiation to homeostasis and the development of diseases [10, 17]. Moreover, organoids have shown potential for high-throughput screening of drugs efficacy and/or toxicity, and to investigate differential gene expression or signalling pathways that differ between normal and abnormal conditions [10, 11, 17-19]. Thus, these models are gradually becoming a promising alternative to more conventional models.

Chapters 2, 4, and **5** describe studies with organoid models of healthy human colon and small intestine (SI) that are exposed to various drugs to generate new data on intestinal drug-induced toxicity. Furthermore, we evaluated whether these models could identify both known and potential novel mechanisms of action of the different drugs tested. In order to expose our *in vitro* cell models to clinically relevant drug concentrations, physiologically-based pharmacokinetic (PBPK) simulations were performed taking into account the dose regimens recommended to patients [20-22]. Since the goal was to study the adverse effects during therapeutic conditions, we ensured that these concentrations were within the therapeutic range. Therefore, we applied concentrations that resemble the drug concentration range that occurs in the human gut epithelial cells upon treatments.

In **chapter 2**, human intestinal organoids were exposed to 10, 100, and 1000 μM of 5-Fluorouracil (5-FU) for 24, 48, and 72 h. 5-FU is a widely used chemotherapeutic agent that is known to cause acute intestinal toxicity. 5-FU is an analogue of uracil, thus it is easily transported into the cells where it exerts its action by incorporation into DNA and RNA, perturbing their synthesis and function. It also interferes with thymidylate synthase (TS) function and stimulates the production of reactive oxygen species (ROS), thus increasing oxidative stress-related toxicity [23, 24]. After organoid treatment with 5-FU, cytotoxicity and functional endpoints were measured. The results demonstrated a time and concentration-dependent response with decreased viability (based on ATP levels), cell size, loss of shape, and increased caspase 3/7 activation, and percentage of cell death. Next, differentially expressed genes (DEGs) (q-value < 0.05) were identified and used for pathway analysis. We found that cell cycle, p53 signalling, mitochondrial ATP synthesis, and apoptosis were among the most strongly affected biological processes after exposure to 5-FU, and that correlated with the functional outcomes. The perturbations in gene expression and biological processes were, overall, stronger in colon than in SI organoids, supporting the hypothesis of a tissue-specific response. This was also demonstrated in the time-series analysis using Short Time-series Expression Miner (STEM), after which specific pathways and DEGs stood out for colon and SI. In colon, more affected pathways were biosynthesis and transport of small molecules, one-carbon metabolism (folate) and

mRNA translation, and the list of more relevant genes comprised *SLC9A3* and *AQP6,* both involved in the transport of small molecules; *JMJD4* (translational termination via ETF1)*, FIBCD1* (endocytosis and inflammation) and *FASTK* (FAS-mediated apoptosis). On the other hand, in SI organoids the strongest affected pathways were cell signalling mediated by Rho GTPases and fork-head box transcription factors, and the most relevant genes included *KIF14, KIF26B* and *SMIM10L2A,* involved in signalling pathways (p-53 and by RHO GTPases); *AQP12B* (transport of small molecules*)* and *TMEM187* (function uncertain). In addition to transcriptomics, metabolomic data was generated by analysing the supernatant metabolome profile of untreated and treated organoids. Overall, TCA cycle and oxidative stress were the most perturbed pathways in both organoid types. Moreover, we reported tissue-specific alterations in the metabolome profile. In colon, a significant depletion of leucine and isoleucine (amino acid metabolism) was observed, whereas, in SI, there was a depletion in lactic acid (inhibition of the glycolytic pathway). After, transcriptomic and metabolomic data were integrated, taking into account that the data sets were derived from organoid pellet versus supernatant. This integrative exercise revealed a potential new mechanism involving factor E2F1 as a regulator of cell cycle and apoptosis. The next step will be to generate intracellular metabolomic data to complement the findings described in this chapter.

Regarding 5-FU mechanisms of toxicity in the intestines, few studies have looked into transcriptomic responses [2]. One rodent study on 5-FU toxicity reported p53-dependent induced apoptosis and inhibition of cell cycle progression [25], similarly to our findings. Additionally, two DEGs found in our study were previously reported in human intervention studies, namely *TYMS* [26] and *EML2* [27]. The first one codes for TS, which generates thymidine for DNA synthesis. Deregulation of *TYMS* leads to an imbalance of deoxynucleotides, and consequently, DNA damage, which is linked to 5-FU mechanism of action. *EML2* is involved in cell *signalling and cell growth. Nevertheless, those two clinical studies included only tumour colon samples and patients received other drugs in addition to 5-FU* [26, 27]. *Therefore, validation of our findings with human clinical data is still rather limited. Currently, one clinical study is looking into the best combinatory therapy with 5-FU including the measurement of potential biomarkers of gut toxicity in the faeces as one of the outcomes [28].*

In **chapter 4**, colon and SI organoids were exposed to 1, 10, 30, and 60 µM of doxorubicin (DOX) for 24, 48, and 72 h. Like 5-FU, DOX is a standard chemotherapeutic agent recommended for several cancers and belongs to the group of anthracyclines [29]. Key events in the mechanism of action of DOX include intercalation into DNA, disruption of DNA topoisomerases, and generation of ROS [30, 31]. Cardiotoxicity is a well-documented side effect induced by this drug, but only a very little number of studies have addressed intestinal toxicity. To fill this gap in toxicity data, we exposed our colon and SI organoids to study DOX-induced intestinal toxicity. The results showed that cell viability tended

to decrease in a concentration and time-dependent manner after exposure to 10 μM in both organoids, whereas caspase 3/7 activation tended to increase in colon but in SI, it remained almost unchanged. Transcriptomic analysis indicated perturbations in cell cycle, DNA repair, p53 signalling pathway, and oxidative stress, all linked to previously known DOX mechanisms of action. DNA methylation was also significantly affected in both organoids, whereas differences between colon and SI organoids were mainly observed in metabolic pathways. Energy generating pathways of glycolysis and lipids metabolism were only found affected in colon. On the other hand, metabolism of amino acids was affected at earlier time points in SI but later in colon. These results suggest potentially different mechanisms being involved in different parts of the intestinal tract. Gene expression analysis using STEM revealed different DEGs affected in colon (*DHRS2, RGCC, LAMP3, TP53I3, TNFSF15, ABCA12* and *MFAP3L*) and SI (*CAPN8, CTNND1, MPRIP, TSPAN1, TPX2, MCM5, DHRS9, SLC2A3, PPP1R3C* and *MT1X*). The proteome analysis applied in chapter 4 identified 19 proteins associated with DOX intestinal toxicity, which are involved in DOX metabolism, cell growth, differentiation and proliferation. Here, the proteomics data was obtained via computational simulations and not directly measured on the exposed cells. Therefore, some proteomic information might not have been fully covered as the *in silico* work was only based on known affected gut tissue proteins. However, for most proteins, the correspondent encoding genes were found significantly affected in the organoids after exposure to the drug. These results led us to conclude that exposure to DOX may result in a strong proliferation inhibition effect rather than apoptotic effect. Future integration of transcriptomics and proteomics generated from exposed organoids may complement the findings reported in this chapter.

Even though clinical studies on DOX intestinal toxicity are lacking, as these mostly focus on cardiotoxicity, we compared the DEGs found in exposed colon and SI organoids with the transcriptomic data available in cardiomyocytes. Interestingly, two of the DEGs observed in the organoids were also found in cardiomyocytes exposed to DOX, namely *CCND1* (cyclin D1) and *TP53I3* (Tumor Protein P53 Inducible Protein 3). Cyclin D1 was upregulated in mice cardiomyocytes [32]. Likewise, the expression levels of *CCND1* in colon and SI organoids increased over time and concentrations of DOX. In turn, the *TP53I3* gene was observed in exposed human-derived cardiomyocytes [33]. Despite not being tissue-specific, these two genes could be relevant in the investigation of gene responses to DOX as they seem to be implicated in cardiotoxicity and intestinal toxicity. Future clinical studies will be valuable in validating gene markers of DOX-induced intestinal toxicity.

Next, **chapter 5** is focused on the investigation of the transcriptomic signatures of gefitinib, a more recent anti-cancer drug than the previous ones. Gefitinib is a tyrosine kinase inhibitor (TKI) and acts by selectively inhibiting the epithermal growth factor receptor (EGFR), and subsequent inhibition of many signalling pathways that regulate cell cycle, proliferation, survival, and apoptosis [34, 35]. Among the most common adverse

effects, gefitinib causes diarrhoea in 30% of patients [34]. Also for this compound, only a limited number of investigations have looked at the effect on intestinal damage. Thereby it demonstrates the need to generate new data that can provide insight into the mechanisms by which TKIs induce toxic side effects in the intestinal tract. In our studies, we exposed colon and SI organoids to 0.1, 1, 10, and 30 μM of gefitinib for 24, 48, and 72 h. Evaluation of cell viability showed that it decreased across time and concentration in both organoids. Caspase 3/7 activation increased after exposure to the two highest concentrations in colon, but only at 30 μM in SI. Morphological changes were different as size and roundness were more significantly altered in SI unlike the percentage of cell death, which was higher in colon. Transcriptomic analysis identified that signal transduction pathways were amongst the most perturbed pathways, in line with gefitinib's mechanism of action [34, 36]. Likewise, apoptosis, FOXO-mediated transcription, p53 signalling pathway, and metabolism, particularly glycolysis, TCA cycle, respiratory electron chain, metabolism of lipids and cholesterol, were affected by the drug. Moreover, genes that encode for cell adhesion molecules E-cadherin and β1-integrin were found downregulated hence contributing to the drug-induced toxic effects [37, 38]. Differences in gene expression were also observed between the organoids. For instance, pro-apoptotic genes *BAX* and *SUSD6* were upregulated in colon but not in SI. Another interesting difference between the organoids was the opposite direction of expression of genes involved in the metabolism of lipids and cholesterol. These genes were mostly downregulated in colon and upregulated in SI. We hypothesized that these differences may be related to different expression levels of *PRKAB1*, which in turn regulates activated protein kinase (AMPK) activity. When activated in stress conditions, AMPK can inactivate key enzymes for the biosynthesis of lipids and cholesterol [39]. Overall, genes and biological processes were more affected in colon organoids, suggesting that the colon may be more sensitive to gefitinib as compared to the SI. In turn, SI organoids seemed to have triggered a mechanism to better resist gefitinib effects by increasing cholesterol synthesis, which has been associated with cell resistance to the drug [40-42].

As far as our knowledge goes, there are no clinical studies with gefitinib addressing intestinal toxicity. Previous *in vitro* studies with cancer cell lines reported upregulation of *BAX* and *BCL2L11* (apoptosis) [43, 44], as observed in our study with the intestinal organoids. Additionally, and similarly to our findings, exposure of mice and human intestinal epithelial cells (IEC-6 and CCD 84441 CoN, respectively) to gefitinib resulted in downregulation of *CCND1* (cell cycle), *CDH1* (E-cadherin), and *ITGB1* (β-integrin) [37, 38].

The transcriptomic data obtained for the colon organoids exposed to 5-FU (**chapter 2**) was compared with data generated from colon biopsies of patients with advanced breast cancer taking capecitabine, a pro-drug of 5-FU (**chapter 3**). This study aimed to investigate gene expression responses and biological processes affected in human healthy colon tissue biopsies after monotherapy with capecitabine and further

evaluate the translatability of the colon organoids model. First, transcriptomic analysis of the colon biopsies showed that the most perturbed pathways were the transport of small molecules, cellular responses to stress, folate metabolism, NF-kB signalling pathway, and immune system responses. The most relevant genes were *ATP12A, SLC26A3* and *AQP8,* involved in the transport of ions and water; *TRIM31*, a regulator of Wnt, p-53 and NF-kB signalling pathways [45]; *MST1P2* and *MST1L,* stimulators of macrophages. The expression changes in the three membrane transporters encoding genes suggest dysregulation in water/ions reabsorption, which leads to diarrhoea [46]. In turn, *TRIM31,* by regulating essential signalling pathways, is involved in cell growth, proliferation and apoptosis. Additionally, and along with *MST1P2* and *MST1L*, *TRIM31* has a role in activating immune system responses and in increasing levels of cytokines [47]. These inflammatory responses seem to be secondary effects of 5-FU, triggered by colon cells damage derived from the exposure to the drug [48]. These events can also suggest the onset of diarrhoea. From a clinical perspective, these genes could be potential candidates to predict diarrhoea induced by 5-FU. Second, the comparison of biological processes affected in the human patients with those of the colon organoids clearly identified an overlap related to the transport of small molecules, cellular responses to stress, and folate metabolism, as these were also affected *in vitro*. Remarkably, *TRIM31* was also found upregulated in colon organoids exposed to 5-FU. Therefore, this gene could represent a promising biomarker of drug-induced colon toxicity *in vitro* that may contribute to the improvement of drug screening studies before clinical testing of new fluoropyrimidines-based medicines. *TRIM31* may also constitute a starting point in investigating translatable gene markers in colon organoids. Further validation of these findings is needed by setting up a larger study, including patients who have and those who do not have diarrhoea as an adverse effect of 5-FU. The inclusion of more patients will also contribute to fully checking the translatability of the organoid model.

An overview of the biological processes affected by the different drugs in the colon and SI organoids, as well as in the cancer patients, is represented in Figure 1. Comparing the molecular pathways and DEGs affected in the intestinal organoids by the different exposures, we can observe some similarities and differences in the responses to the drugs. Due to their distinct mechanism of action, different outcomes were observed, mainly in the transport of small molecules, respiratory electron chain and ATP synthesis, activation/inhibition of signalling pathways, apoptotic or cellular senescence events, cell adhesion, and metabolism of lipids and cholesterol. Concerning the differences between *in vitro* and *in vivo*, these rely on perturbations in inflammatory responses and NF-kB signalling pathway observed in the patients' samples, similar to a previous animal study [49]. Looking at the gene expression level, each drug statistically significantly affected a different set of DEGs, as well as specific genes in colon and in SI.

Although our results point out several different molecular events and DEGs, the drugs' effects cause perturbations in cell cycle progression and energy production

processes, mainly glycolysis and TCA cycle, activated p-53 signalling pathway, and oxidative stress (except for gefitinib). Furthermore, aquaporins and membrane transporters were affected *in vitro* and *in vivo*, suggesting the same mechanism of toxicity, despite the encoding DEGs being distinct. Therefore, it seems that different pathways underlying each drug-induced toxicity culminate in the same biological processes that affect cell growth, proliferation, differentiation, ultimately contributing to intestinal damage and diarrhoea.

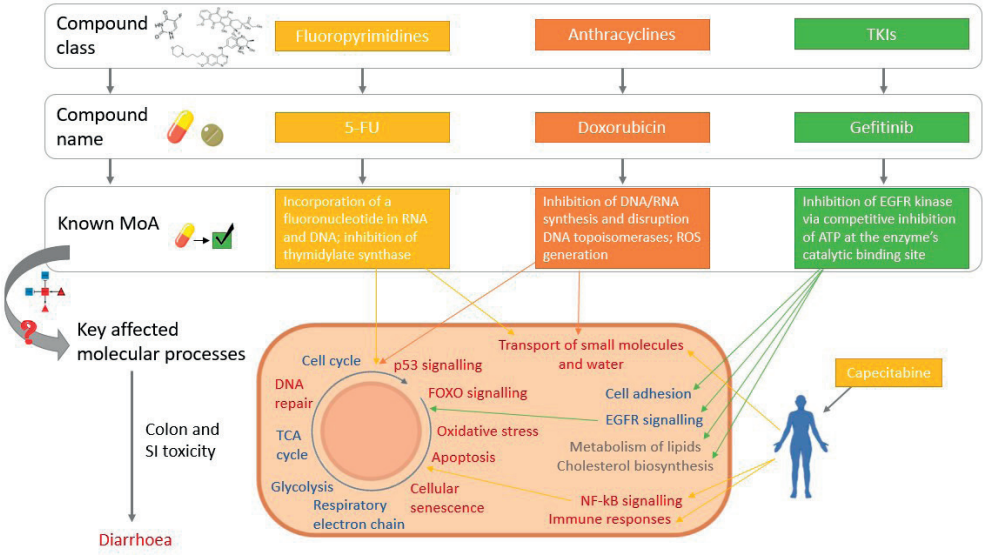

Figure 1. Overview of the key molecular processes affected in the colon and SI organoids by the different drugs selected to investigate intestinal toxicity. Molecular processes affected in the colon biopsies of the cancer patients receiving 5-FU are also included. Consequently, intestinal toxicity can lead to diarrhoea. Pathways in blue are inhibited by the drug, in red are activated and in grey both depending on the type of organoid. Legend: MoA, mechanism of action.

The organoids models implemented in this work surely offer cellular traits that other *in vitro* models do not have. However, the absence of immune cells is a limiting factor in the comparison between *in vivo* and *in vitro* toxicity evaluation. Immune cells are important for the maintenance and homeostasis of SI and colon tissues, including residential colonic macrophages [50]. It is well documented that inflammatory processes can also play a role during adverse events in many tissues, such as in the liver [51], skin [52], eye [53], and the gastrointestinal (GI) tract [54]. Thus a co-culture model of macrophages and colonocytes may allow us to study the impact of immune cell-mediated toxic responses induced by pharmaceutical compounds. For this reason, we have established a new and promising 3D co-culture system combining the human colon organoids and THP-1 derived macrophages (Mφ), which is described in **chapter 6**. In the first experiments with the co-culture, this *in vitro* system was exposed to lipopolysaccharide (LPS), a known activator of inflammatory responses; DOX, known to cause intestinal toxicity; and the combination of

DOX with ibuprofen, a non-steroid inflammatory drug (NSAID) that reduces inflammation. Compared to the mono-culture of colon organoids, the co-culture showed a significantly higher production of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β upon exposure to LPS and DOX. The addition of ibuprofen decreased the levels of cytokines in the co-culture to similar levels as observed in the controls, suggesting that ibuprofen decreased the inflammatory response. These results led us to conclude that interaction between colon organoids and Mφ triggered different inflammatory responses, based on cytokine production and image analysis. The co-culture model that we established might hence constitute a promising tool to investigate further inflammation-associated drug toxicity. Still, future studies are needed to fully explore the new *in vitro* model. These include the investigation of gene expression changes and biological pathways associated with colon-Mφ interaction and its role in responses to drugs.

Future innovations to improve the translatability of *in vitro* toxicity testing

This work has brought innovative *in vitro* models to study drug-induced intestinal toxicity and generate new data, particularly on transcriptomic signatures. Nevertheless, there is still room for advancing the *in vitro* models for toxicity testing and their translatability.

The combination of the colon and SI organoids models with residential macrophages [50, 55], and other immune cells [56, 57] in a co-culture system is a remarkable innovation. These immune cells are important for the maintenance and homeostasis of the tissues. The immune system also plays an important role in the onset of inflammatory processes triggered by exposure to compounds [54]. For these reasons, this thesis includes the development of a co-culture model as a starting point to advance the colon organoid model and the investigation of drug effects on the onset of tissue inflammation. Likewise, the intestinal organoid model would benefit from the addition of the microbiome, which can also affect the responses towards drugs [58-61]. It has been demonstrated that the microbiome contributes to the homeostasis of the intestinal epithelium, being involved in gut metabolism, nutrition, physiology, and immune responses [62, 63]. Although there have been already some advancements in generating gut-microbiome cultures, the full understanding of host-microbe interplay, microbiota's genetic traits, and flux rates of microbiome-derived metabolites is still lacking [64, 65]. Other challenges comprise the combination of strictly anaerobic microbes with colonic cells, which require oxygen to grow [64], and the diversity of microbe strains that can vary per individual [65].

An additional advancement would be overcoming the challenge of the donor variability of the colon and SI organoids generated in this thesis. Future studies including paired healthy colon and SI organoids would offer various advantages regarding the

validation of tissue-specific drug responses, as well as the exclusion of the potential impact of donors' genetic background on relevant drug-gene responses. Nevertheless, it is challenging to generate colon and SI organoids from the same individual because donors would have to undergo unnecessary surgical procedures. Intestinal paired organoid models are not commercially available either yet.

Concluding remarks and future perspectives

This thesis demonstrates the value of using advanced *in vitro* models and experimental methods in toxicogenomics. We established a 3D cell culture organoid model of colon and SI tissues that were exposed to physiologically relevant drugs' concentrations to better reflect the human *in vivo* conditions. A high-throughput transcriptomic approach was also applied to gain insight into gene expression changes specific to each drug. New transcriptomic data was generated from different classes of chemotherapeutics, addressing the lack of data on intestinal toxicity compared to other tissues. As a result, this work contributes to the better understanding of relevant pathways affected by drugs. The main outcomes of the different exposures are summarized in Figure 1, which highlights novel and different molecular mechanisms associated with each drug. Cell cycle and p-53 signalling pathway are common pathways perturbed by the drugs. This suggests that drug-specific molecular pathways and genes lead to cell cycle arrest. From a clinical toxicity perspective, these results created new avenues for the distinction of SI from colon toxicity, and in finding targetable genes to prevent, monitor, or attenuate drug adverse events.

Furthermore, the development and application of the intestinal organoids model and experimental design applied in this thesis can improve *in vitro* drug toxicity assessment studies. Potentially, the *in vitro* model could become an alternative to conventional *in vitro* and animal models in predicting human drug responses. Despite this, there is still room for improvement of the intestinal organoids model by combination with immune cells and microbiome to more closely replicate the human gut environment in healthy and disease conditions. The advanced approaches used in this thesis and proven successful, and their further exploration, will stimulate the future of toxicity research.

The work described in this thesis also represents a step forward in the development of safer medicines and a promise in improving translatability between the organoid model systems and human subjects. Noteworthy, the new transcriptomic data presented in this thesis will be integrated into a quantitative systems toxicology (QST) model to predict GI toxicity from different classes of compounds. In this sense, this will be a fundamental contribution to the Translational Quantitative System Toxicology (transQST) project [22] in developing new tools to improve drug safety assessment during pre-clinical studies. Finally, this work will open doors to the investigation of toxicity biomarkers that will aid in the prediction of clinical outcomes and improve the monitoring of adverse events.

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