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Complications of Treatment

Drug-induced gene expression profile changes in relation to intestinal toxicity: State-of-the-art and new approaches

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ABSTRACT

One of the major complications that patients experience during pharmacological treatment is the occurrence of adverse drug reactions (ADRs). The most affected organs are the liver, kidney, heart and the gastrointestinal-immune system. In comparison to the other organs, less progress has been made on human-relevant prediction of drug-induced intestinal toxicity, evidencing current large data gaps. The most widely used drugs that are associated with intestinal damage include chemotherapeutics, such as 5-Fluorouracil or Tyrosine Kinase Inhibitors (TKIs), as well as non-steroidal anti-inflammatory drugs (NSAIDs). Chemotherapeutics are regarded as inducers of acute intestinal toxicity whereas NSAIDs are associated with chronic inflammation of the intestine. In view of the fact that only a few studies have been dedicated to studying cellular and genomic responses in relation to drug-induced intestinal ADRs, little is known about how intestinal toxicity develops after exposure to such drugs or which molecular mechanisms are involved. Therefore, new models and experiments are required to establish transcriptomic responses and alterations of molecular markers induced by different medicines. This review summarizes the available information about transcriptomic responses and biomarkers of toxicity induced by 5-FU, NSAIDS or TKIs in different experimental models. Future investigation should address the challenges in predicting intestinal toxicity induced by drugs and unveil specific gene expression profiles that can be applied in the development of safer drugs.

Background on intestinal toxicity

The intestines are one of the organs that compose the lower gastrointestinal (GI) tract, being mainly responsible to turn food into nutrients and energy, expelling the waste as faeces. The intestines present a distinctive architecture that supports the important and complex functions of this organ and they are divided into small and large intestine. Each part presents a unique structure and metabolic activity [1], has a different composition of cells [2], expression of genes/proteins [3] and composition of the microbiome [4]. Intestines exert highly important functions, being a key organ not only in the absorption and metabolism of nutrients, drugs and xenobiotics, but also as a defensive barrier, as part of the immune, endocrine and neuromotor systems [1]. Due to their important role in our organism, intestinal injury has become an increasing concern among clinicians, particularly because toxicity can be dose-limiting in several drug based therapies, mainly related to anticancer agents and nonsteroidal anti-inflammatory drugs (NSAIDs), leading to adjustment, reduction or interruption of the therapy [5,6] (Fig. 1). This problem can severely affect patients' health not only due to the risk of developing intestinal toxicity, but also because their treatment becomes compromised.

Intestinal damage caused by therapeutics and other xenobiotics is commonly referred as mucositis, a term introduced in 2006 by Medical Subject Headings (MeSH) [7]. Mucositis consists in damage of mucous membrane and loss of mucosal integrity, thus compromising the body's main barrier against infections, and predisposing patients to inflammation, higher risk of developing bacterial, fungal or viral infections, and ultimately, sepsis, particularly in already immunosuppressed patients [8]. Intestinal mucositis is an extremely common adverse effect of cytotoxic therapies, affecting approximately 40% of patients undergoing standard chemotherapy regimens [7]. Chemotherapeutic agents destroy the continuously dividing mucosal cells of the intestines, and if cells are not given enough time to quickly renew themselves, inflammation processes arise, which may lead to ulcerations and alteration of mucosal barrier functional integrity [9]. More specifically, cancer-therapy induced mucositis may result in apoptosis of intestinal

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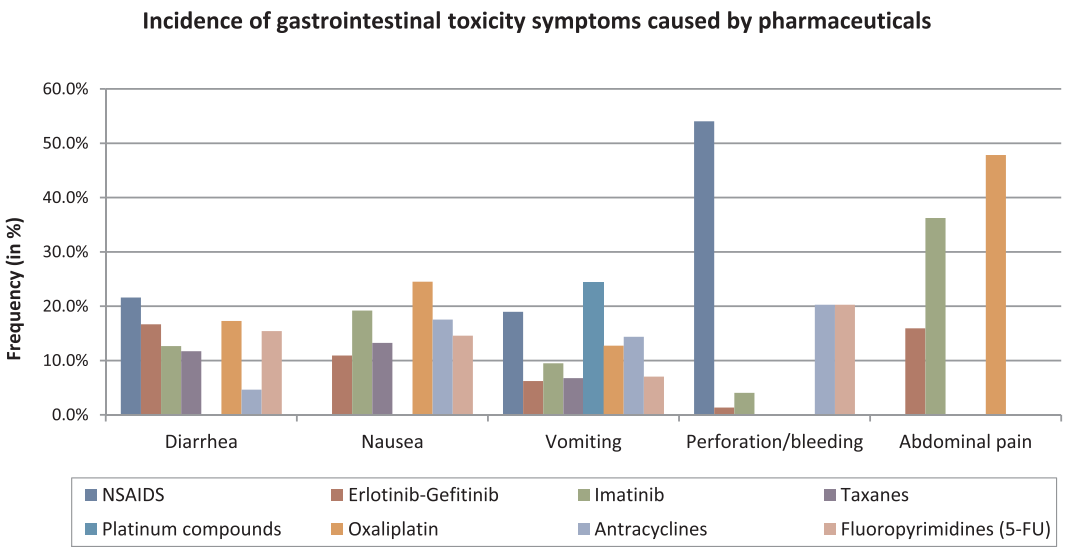

Fig. 1. Distribution of the main adverse effects associated with drug-induced intestinal toxicity. NSAIDs, fluoropyrimidines (5-FU) and oxaliplatin are among the pharmaceuticals that cause more intestinal problems to patients. Figure was generated based on data presented in references [13,14].

crypt cells and regenerative stem cells, villous atrophy and reduction of enterocyte mass [3,9]. Clinical common symptoms include nausea, vomiting, abdominal pain, constipation and diarrhoea with blood or mucus, leading to a decrease in the absorptive capacity of intestines [5,10]. Clinical consequences generally comprise dehydration, malnutrition, ulceration and perforation [5,11], increasing the risk of general complications, such as infections and even mortality [11]. This affects the absorption of orally administered pharmaceuticals [2] and hinders the efficacy of the correspondent therapies. The extent and severity of intestinal toxicity and its consequences depend on several factors such as dose, route and time of exposure, frequency of therapy administration, combinations with other types of therapeutics and the ability of the intestinal mucosa to regenerate from damage [12]. Furthermore, factors related to individual characteristics, either genetic or life-style factors, can also determine the severity of intestinal toxicity, even for patients taking the same therapy [7].

Current diagnostic methods of intestinal toxicity are quite limited, lack specificity, are mostly invasive and cause discomfort to patients [5,7]. On the one hand, symptoms experienced by patients are non-specific, thus being an unreliable measure of intestinal toxicity [15]. On the other hand, evaluation of intestinal damage is challenging since it is not easily observed or detected without relying on invasive procedures. The current gold standard method to assess intestinal toxicity is histopathology [2], which relies on endoscopy, with or without biopsy, thus being invasive and presenting a risk of bleeding or other complications [15]. In addition, it might fail to identify focal or regional injuries without extensive sample collection [2]. There are also a few non-invasive tests, such as sugar permeability tests [16], 13C sucrose breath test and monitoring of diarrhoea/constipation, but they all have limitations and lack specificity and reliability [5]. The lack of a reliable, specific and validated tool to assess and measure intestine toxicity is evident and, consequently, available information is not consistent enough yet to allow both the development of clinical studies for treatment and management of this condition. Therefore, the possibility of predicting patients' risk to develop intestinal toxicity could potentially improve the quality of therapy, which then could be addressed according to each individual situation [17].

Ideally, a biomarker of intestinal toxicity evaluation should be sensitive, non-invasive, easily assessed by clinicians and quantified [18], early predictive and disease specific [2]. It should also rapidly identify any changes related to intestine disease or injury, reflecting both the different stages of the development of intestine toxicity and the stages of recovery [2,7]. Furthermore, a toxicity biomarker should give information on the disease independently of patients characteristics, metabolic activities, presence of other pathologies, or medication [18]. Blood, urine saliva or faeces are among the main sources of biomarkers due to their rather easy collection and analysis [7]. Nevertheless searching for intestinal toxicity biomarkers has been challenging and only a number of biomarkers has already been evaluated, such as diamine oxidase (DAO), calprotectin, granulocyte marker protein (GMP), CD64, gastrin, citrulline and thymidylate synthase (TS) [7,10,19–21], which can be found summarized in more detail in Table 1. The application of these biomarkers in toxicity screening during drug design and development or in clinical diagnosis and monitoring is still limited because they correlate poorly with histologic evidence of intestinal toxicity or still require validation [19]. Indeed, and despite preclinical success rates having increased over the last 10 years, from 66% to 88%, as well as of phase I and II clinical trials [22,23], the reason for most failures remains related to safety and toxicity issues, in which the gastrointestinal system is among the most affected organ systems (Fig. 2), particularly in clinical trials [23]. This data clearly supports the urgent need to develop more specific and sensitive biomarkers that could work as predictors of intestinal toxicity to improve the safety of medicines, either those which are already on the market or those being developed. Only then, unforeseen intestinal toxicity induced by drugs can be avoided.

Measurement of gene expression after exposure to drugs has gained importance since it may provide information about regulation of genes and biological processes in response to that exposure. The transcriptomic analysis will reflect a particular pattern of genes that can be associated with drug-induced toxicity, providing a more sensitive and specific panel of biomarkers as well as insight in the mechanistic aspects of toxicity. In addition, traditional drug toxicity biomarkers are often detected when the damage has already been induced whereas gene expression changes may occur instantly, allowing a more efficient prediction of intestinal injury. Despite the promise that gene expression profiling has demonstrated in biomarker investigation [24–27], and in the understanding of the development of diseases and personalized medicine [28,29], a lack of studies that address drug-induced gene expression responses is still evident.

Another important aspect that should be taken into account when developing intestinal toxicity biomarkers or therapeutic targets and new medicines, is the heterogeneity between small and large intestines, which is quite often underestimated. As mentioned earlier, the small and large intestine differ in composition, structure and, consequently, in their specific functions, which also reflects on their susceptibility to

Table 1
List of candidate biomarkers for GI toxicity that have been investigated so far in which their main characteristics, advantages and disadvantages are summarized.

Candidate biomarker	Characteristics	Advantages	Disadvantages
Blood samples			
C-reactive protein (CRP) [10,20]	Acute phase protein of inflammation used in clinical practice	Levels correlate well with tissue injury	Non-tissue specific; influenced by other factors; depends on the onset of inflammation
CD64 (neutrophilic) [19]	Proposed biomarker for intestinal inflammatory and functional diseases	Significantly higher in patients with inflammatory bowel disease (IBD); possible distinction between GI disorders	Only mouse antibodies are available so far
Citrulline [7,10,19,20]	Non-protein amino acid; intestines as primary source (produced by enterocytes)	Sensitive, accurate and specific in reflecting enterocyte mass loss; economical; simple to measure	Invasive; requires repeated sampling
Cytokines (TNF-α, IL1-β, IL-6) [7,10,20]	Pro-inflammatory proteins	Markers of inflammatory response; economical	Non-specific; high variability and time-dependency of levels in mucosal barrier injury
Diamine Oxidase (DAO) [10,19]	Highly degradative enzyme involved in polyamine metabolism; localized in mature villus epithelial cells	Plasma activity as potential candidate of intestine injury; high levels in epithelial cells of small intestine	Fast clearance
Gastrin [19]	Peptide produced by endocrine G cells in response to stimuli derived from digestion	Increased levels reflect GI damage	Mechanism of release from G cells not fully understood
Prohepcidin [7]	Acute phase protein; key role in regulating iron absorption in intestines	Increased levels during inflammation; relates to mucositis	One study with small cohort
Faecal samples			
Calprotectin [7,10,19,20]	Non-glycosylated Protein; major soluble cytosol protein in neutrophil granulocytes	Highly sensitive biomarker of intestinal inflammation; correlates well with severity of inflammation; non-invasive; economical	Lack of specificity; does not indicate the site of damage in the intestine
Granulocyte Marker Protein (GMP) [7,10]	Physiologically similar to calprotectin	Sensitive and non-invasive	Non-specific
Transferrin [7]	Plasma glycoprotein; binds to iron reversibly	Levels increase with GI damage	Limited number of studies
Urine or breath samples			
Sucrose breath test [7,10,19,20]	Possible marker of digestive enzymes, enterocytes and function of small intestine	Correlates with mucosal barrier damage; economical	Requires specialized equipment; repetitive and time-consuming sampling
Sugar permeability test [7,10,20]	Non-invasive test; assessment of the function of intestine barrier	Measures gut permeability and absorption; non-invasive; economical	Not routinely used in clinical practices; repetitive; time-consuming sampling

drug-induced toxicity [30]. Digestion and metabolism of compounds can occur with some extent in the small intestine but that does not occur in the colon, which can make it more prone to damage. Additionally, differences in the population of immune cells and bacteria [4,30] that have adapted to each region of the intestines can also influence the likelihood of developing certain diseases such as chronic inflammations or cancer. For instance, Celiac disease only develops in the small intestine whereas ulcerative colitis is specific to the colon [30]. Likewise, this heterogeneity within the intestines will dictate the capacity of both organs in metabolizing xenobiotics/drugs, the responses towards the interaction with those compounds and subsequent development of toxicity. Indeed, the role of the microbiome of both organs has become an aspect of interest over the last years. The number of studies considering the microbiome has grown and interesting outcomes have been described related to drug metabolism and toxicity [4,31–40].

In the following sections, we describe and evaluate intestinal toxicity induced by 5-FU, TKIs and NSAIDs, as these compounds are already

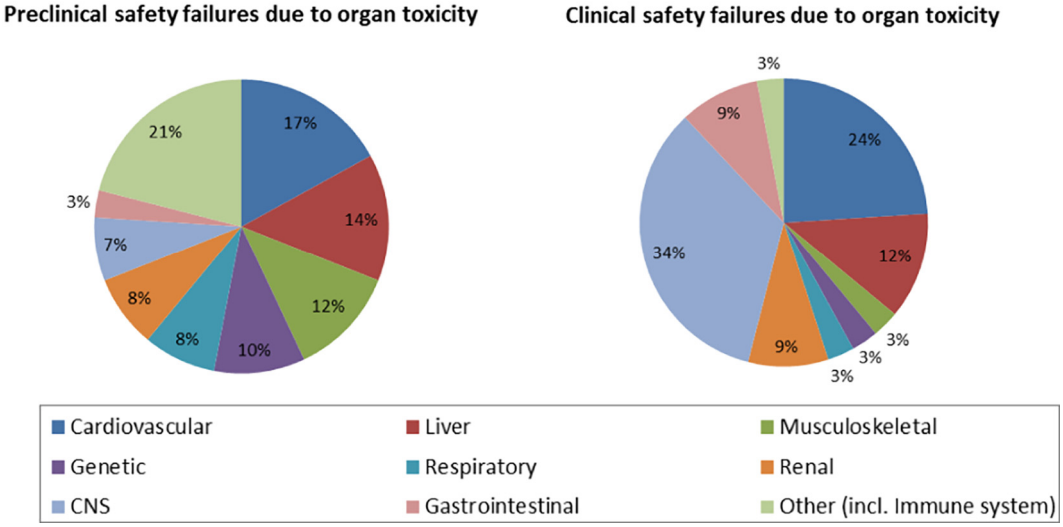

Fig. 2. Safety failures of preclinical and clinical trials due to organ toxicity, including the cardiovascular, liver, respiratory, renal, gastrointestinal and central nervous systems. Figure was generated based on data obtained from AstraZeneca report on R&D productivity [23]. CNS, central nervous system.

highly associated with adverse effects in the intestines, focusing on transcriptomic responses available in different toxicity models (*in vitro*, animal models or human samples). Biomarkers that have shown potential in predicting and diagnosing intestinal toxicity will also be discussed.

Case Studies: anti-cancer drugs

5-Fluorouracil-induced intestinal toxicity

5-Fluorouracil (5-FU) is widely used in the treatment of several cancer types and clinical studies demonstrate that responses to 5-FU vary among patients, evidenced by the fact that some of them display none or mild side effects whereas others present severe adverse reactions (AR) [41]. General common side-effects caused by 5-FU include myelosuppression, dermatitis, cardiac toxicity and mucositis [41,42], being the latter the more serious one that can affect the intestines of 40–80% of patients taking this drug [7,43]. Intestinal mucositis usually results in nausea, abdominal pain, vomiting and diarrhoea. This has a major impact on health and quality of life of patients, and increases the risk of other complications (e.g. fatigue, loss of weight), leading to the discontinuation of the cancer therapy or alteration to a less effective one and even of mortality [12]. Patients suffer from intestinal mucositis due to the fact that 5-FU, as an oncological agent, kills mucosal cells at a higher rate than their renewal capacity. The severity of intestine injury caused by 5-FU depends on factors such as dosage, route and frequency of administration, simultaneous radiotherapy and regeneration capacity of the intestine.

The overall mechanism by which 5-FU exerts its effects is based on incorporation of a fluoronucleotide into RNA and DNA along with inhibition of thymidylate synthase (TS), a nucleotide synthetic enzyme [21]. Being an analogue of uracil, 5-FU can enter the cells easily through the same mechanism of transport as this nucleotide. Afterwards, it is converted intracellularly into many active metabolites that disturb RNA synthesis and TS function [21,44]. Several factors have been associated to 5-FU-induced intestinal toxicity, from reactive oxygen species (ROS) to production of pro-inflammatory cytokines (IL-1β, IL6, TNF-α) and platelet-activating factor (PAF) [45,46]. Nevertheless, key factors, especially transcription factors that regulate genes involved in the development of toxicity, are not well understood yet. Whichever the mechanism of action of 5-FU, it can cause intestinal toxicity and currently, there is no easy, non-invasive and reliable method available to detect it. Clinicians still rely on endoscopy and biopsies procedures, which can cause discomfort to patients or worsen the state of the intestinal mucosa, as well as on patients' own reports about symptoms they might experience. Therefore, discovering biomarkers that can be used as tools to predict intestinal toxicity during the development of chemotherapeuticals or to assess early onset of toxicity in cancer patients that are at risk of developing more severe intestinal injury is required.

In vitro studies

There are a few studies on 5-FU transcriptomic responses established *in vitro* using human colonic cells to determine gene expression profiles that can be associated with intestinal toxicity.

Firstly, Maxwell et al. identified changes in the transcriptomic profile of cells after exposing them to 5-FU, using a cDNA microarray technology [47]. The initial study was performed in a breast cancer cell line MCF-7, but further validation of the results was performed in colon cancer cell lines H630 and H630-R10 (5-FU-resistant). These cells were exposed to 10 μM 5-FU for 72 h. The validation study identified 5 novel 5-FU-inducible transcriptional targets, namely SSAT, MAT-8, Chaperonin-10, Annexin II and Thymosin-β-10 [47]. They were all upregulated in both colon cancer cell models, especially SSAT and MAT-8. Furthermore, the upregulation of these 5-FU-inducible transcriptional targets was more evident in 5-FU-resistant colon cancer cell line. As a result, the authors hypothesize that this panel of biomarkers can potentially indicate cases of resistance to this drug. In addition, p53 seems to play a role in the regulation of those genes.

In another study, two human colon cancer cell lines, HCT-C18 (TS-) and HCT-C18 (TS+), were used to determine the gene expression profile affected by both thymidylate synthase (TS) and 5-FU [48]. Thymidylate synthase (TS) is an enzyme responsible for the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), the only reaction that provides the *de novo* source of thymidylate required for DNA replication and repair [21]. Therefore, this enzyme is an important target in cancer-therapy and a known biomarker of 5-FU toxicity during chemotherapy [21]. It was observed that different genes were affected between TS dependent and independent toxicity induced by 5-FU exposure. Therefore, 5-FU might exert its toxic effects through different mechanisms. Regarding genes affected by 5-FU treatment in a TS independent manner, the expression of 11 genes was found altered, mostly related to cell cycle growth/proliferation, apoptosis, DNA replication/repair, nucleotide metabolism and epidermis and ectoderm development [48]. A similar study was performed using human colon cancer cell HCT116 and 2 resistant cell lines derived from HCT116 [49]. Evaluation of the alterations in expression levels of DNA damage response, cell cycle and apoptosis-regulatory genes unveiled, as expected, that the resistant cell lines were less affected than the parental one by 5-FU effects. After 24 h exposure to 5-FU, alteration in several genes profiles was observed in HCT116 cells. Again, most of the altered genes were related to DNA damage response/repair, cell cycle growth/proliferation, apoptosis, nucleotide metabolism, as well as mRNA processing/transport and amino acid, protein, carbohydrate and oxygen metabolisms [49]. Similar genes were found altered in another study performed by Boyer *et al.* (2006), in which the same human colon cell line (HCT116) was used [50]. More recently, Hou et al. studied the effect of 5-FU in the human colorectal carcinoma cells HT29s [51]. However, this study focused on the microRNA (miRNA) profiling after exposure of cells to 5-FU and starvation (autophagy) to observe which genes would modulate autophagy in 5-FU-based therapy in colorectal cancer (CRC). Overall, four miRNA were found upregulated (miR-302a-3p, miR-548ah-5p, miR-113b, miR-323a-3p) and 27 were found downregulated, being all involved in autophagy processes and seen as important modulators of autophagy in response to events of cellular stress [51].

Animal model studies

Studies on the toxic effects of 5-FU in the intestines using animal models were started in 1998 by Pritchard et al. [52]. A murine model was used (BDF1 mice, and p53 wild-type (+/+) and p53 null (−/−) mice of both sexes), in which either 40 or 400 mg/kg of 5-FU was administered. This study aimed at establishing a relation between *p53* expression and 5-FU induced apoptosis and inhibition of cell cycle progression in intestine cells. Indeed, a p53 dependency was observed coupled to 5-FU toxicity responses, including cell apoptosis, inhibition of proliferation and loss of crypt and villus integrity [52].

Additionally, Chang et al. observed that 5-FU affected mainly genes regulated by NF-kB factor after intraperitoneally administration of 100 mg/kg in mice [41]. The results led the authors to conclude that NF-kB is the central molecule in 5-FU-induced intestine toxicity, being its activity significantly increased by this drug. Expression levels of IL-6, TNF-α, IL-1β were also found increased in response to the exposure to 5-FU [41].

Kalabat et al. aimed at investigating miRNAs as potential biomarkers of intestine toxicity [2]. Three rodent models of drug-induced intestine toxicity were included to evaluate effects of three different drugs by analysing plasma and faeces. Although 5-FU was not one of the drugs included, effects of PAK4 and HSP90 inhibitors included in this study might be similar to 5-FU since they all are anti-proliferative compounds. The most promising toxicity biomarkers observed were

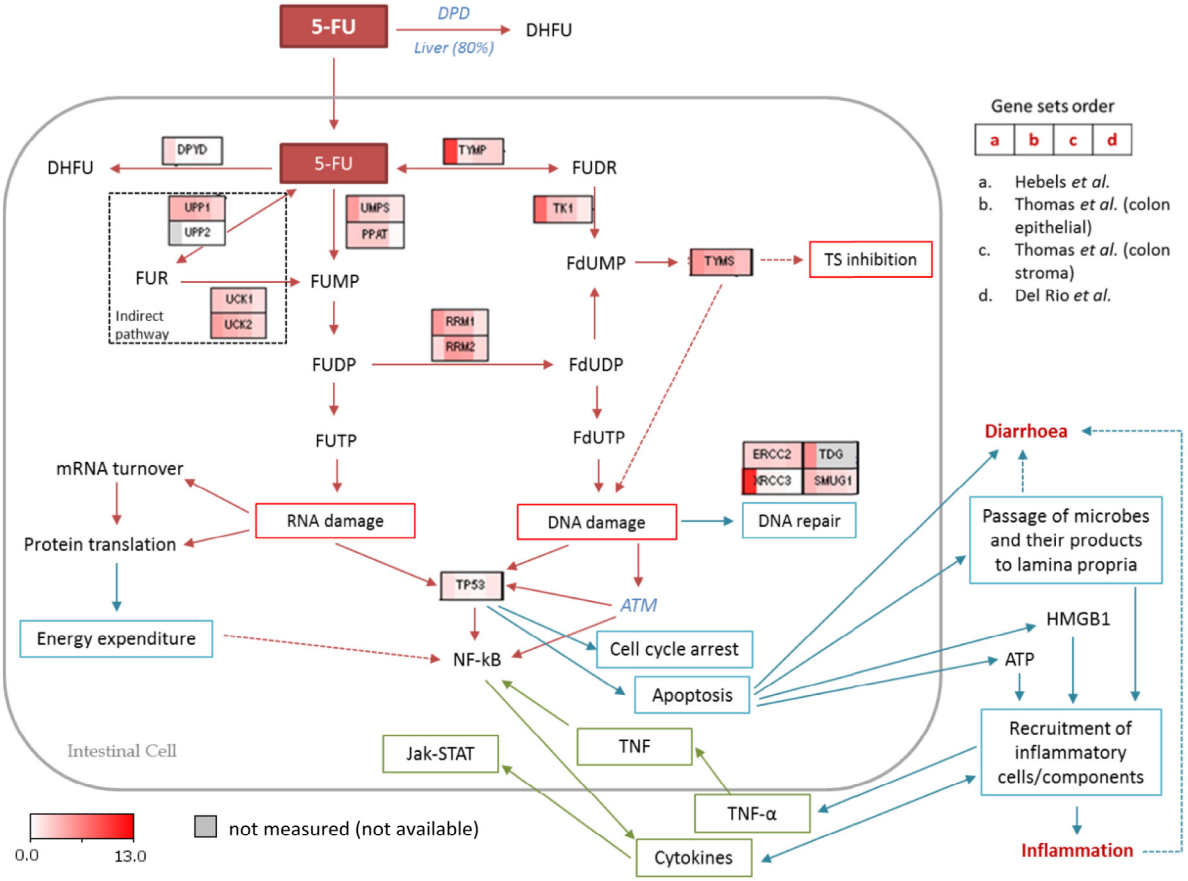

Fig. 3. Schematic representation in PathVisio [61] of 5-FU general metabolism into its main products: FdUMP, FdUTP and FUTP, in intestinal cells. Genes that code for enzymes responsible for 5-FU metabolism and activity are represented considering the baseline gene expression based on Bikel et al. correction [58] for the y-chromosomal gene expression in females (correction for log2 intensities). Conversion of 5-FU into DHFU by DPD is the rate-limiting step. Approximately 80% of 5-FU is metabolized by DPYD in the liver. 5-FU target points of action that are considered to be responsible for the symptoms of toxicity (diarrhoea) and inflammation, namely TS inhibition, DNA/RNA damage, cell cycle arrest, apoptosis and production of cytokines, are also represented. Legend: *Metabolites:* FdUDP, fluorodeoxyuridine diphosphate; FUDP, fluorouridine diphosphate; FUDR, fluorodeoxyuridine; FUMP, fluorouridine monophosphate; FUR, fluorouridine; FUTP, fluorouridine triphosphate. *Enzymes:* ATM, serine/threonine kinase; DPD, dihydropyrimidine dehydrogenase. *Genes*: grey - not measured (not available); white - expression below threshold (set to zero); red - expression is above threshold (max. 13). The gene set order is as follows: a. Hebels et al. [59]; b. Thomas et al. (colon epithelial) [60]; c. Thomas et al. (colon stroma) [60]; d. Del Rio et al. [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

miR-215 and miR-194, being both increased in faeces after drug exposure. In plasma, miR-215 was decreased but miR-194 was not detected [2]. Authors also suggest a relation between plasma miR-215 and plasma citrulline. In a similar study, in which toxic effects of PAK4 inhibitors were evaluated as well, miR-194 and citrulline were identified as the most promising intestinal toxicity biomarkers [19]. Even though 5-FU was not included in both studies, the results may indicate miR-215, miR-194 and citrulline as potentially relevant markers of 5-FU induced toxicity.

Unfortunately, additional studies on the effects of 5-FU in animal models do not focus on gene profile alterations. For instance, Manzano et al. studied 5-FU-induced intestine toxicity in pigs (6 healthy and 6 treated with 5-FU). After 4 weeks of treatment, samples from jejunum, ileum and colon were collected to analyse histological, immunological and biochemical modifications. Concerning the colon, a decrease was observed in the colon mucosal weight and thickness, in protein, DNA and GSH mucosal contents, in the enzymatic antioxidant defence system and in the number of lymphocytes [3]. In two other studies with mice, a more targeted approach was performed. In one of the studies, the aim was to evaluate the levels of DAO in response to 5-FU [53], which was decreased in both plasma and mucosa. In the other, effect of 5-FU was evaluated in immune system cells [54]. A decrease in the population of Th1 cells (antitumor immunity) but increase in the population of regulatory T cells and specific activation of CD8+ T cells was observed. Furthermore, an increase occurred in the production of inflammatory mediator nitric oxide (NO) in intestine cells [54], which might suggest another putative biomarker regarding 5-FU toxicity.

Studies in humans

Few attempts have been made to investigate a gene expression signature of intestinal toxicity induced by 5-FU. Nevertheless, there are two major setbacks that hamper any conclusions regarding transcriptomic responses to 5-FU exposure. First, patients included in these studies did not receive solely 5-FU but were exposed to a combination of drugs, including 5-FU, as part of their cancer therapy regimen. Second, intestinal samples were analysed only after the exposure to the drug, thus no data is available on human samples before the exposure in order to establish alterations in gene expression profiles between pre- and post-treatment.

Rio et al. included tumour colon samples from 20 advanced colorectal cancer patients and 18 normal tissues from the same patients, who were being treated with the FOLFIRI regimen, which includes a combination of leucovorin, 5-fluorouracil and irinotecan. A final set of 14 predictive genes was found, being all upregulated [55]. These genes are involved in several different biological functions, such as nucleotide binding, RNA/DNA binding, transporter activity, receptor binding/

activity [55]. However, as the study does not include monotherapy with 5-FU, conclusions about any gene expression changes induced by the drug cannot be drawn.

In another human study, a set of 10 target genes known to be polymorphic with respect to toxicity outcomes were evaluated to determine if they were indeed associated with intestinal toxicity induced by drugs [17]. This is limited in the sense of not providing insight into a more complete altered gene profile as a consequence of drug toxicity in the intestines. Furthermore, only blood samples from patients with advanced CRC were collected, in addition to being treated with a clinical trial treatment regimen (FOCUS trial). The FOCUS trial includes and evaluates different combinations of anticancer drugs (5-FU, irinotecan and oxaliplatin), and 5-FU monotherapy as well. However, 5-FU monotherapy did not show significant associations between gene polymorphisms and severe 5-FU-induced toxicity [17]. Later on, the same authors suggest, in a review paper, 5 promising molecular markers, once more associated with polymorphisms that might contribute to the prediction of 5-FU-induced toxicity. These include *DPYD*, *TYMS-ER*, *TYMS-1494, C677T MTHFR* and *MTHFR 1298 C* [56]. For instance, polymorphism of the gene *DPYD* is associated to cases of deficiency of Dihydropyrimidine dehydrogenase (DYPD), an enzyme responsible for pyrimidine degradation, which prompts cancer patients to more severe toxic effects in the intestines caused by 5-FU [53].

As far as our knowledge goes, there are no more studies investigating *in vivo* 5-FU-induced gene alterations that can be associated with its toxic effects in the intestines. Studies that attempted to search for possible gene profile signature of 5-FU toxicity present some limitations, as described above. Therefore, it is evident the lack of information related to this issue, which demonstrates the need to generate new data on intestinal toxicity caused by this compound. In addition to investigate alterations in the gene profile after exposure to 5-FU, it is also important to better understand the mechanism of action that lead to the adverse effects associated to this drug. Only then, a predictive tool combining cellular and mathematical models can be generated to improve the development of new cancer therapies with similar mechanisms as of 5-FU, so that they cause no further harm to cancer patients.

Despite the fact that there is little data on transcriptomic-signatures of 5-FU, an attempt was made to visualize relevant toxicological pathways (Fig. 3) based on already known information about 5-FU activity [21,57]. Fig. 3 shows the general metabolism of 5-FU into its main products, namely fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), fluorouridine triphosphate (FUTP) and dihydrofluorouracil (DHFU). Factors such as p53 and NF-kB seem to play an important role in mediating gene responses, particularly in genes involved in cell cycle arrest, apoptosis, ATP production and recruitment of inflammatory components including cytokines and TNF-α, which are related to symptoms of toxicity (diarrhoea) and inflammation (Fig. 3) [41,52].

Baseline expression of the genes involved in 5-FU metabolism and activity was obtained using the approach of Bikel et al. [58] to obtain the absolute gene expression from human microarray data by correcting for the y-chromosomal gene expression in females, and visualized in Fig. 3 to show that most of the genes seem to be already above threshold. Furthermore, these genes are expressed differently depending on colon regions and cell types. For instance, genes that code for enzymes responsible for the metabolism of 5-FU (e.g. *TYMP*, *UMPS, TK1* and *DPYD),* seem to be more expressed in the rectum [59] followed by the sigmoid colon [60] and descending colon [55]. Consistently, genes involved in DNA repair (*XRCC3*, *TDG* and *SMUG1*) are more affected in the rectum compared to the rest of the colon. Therefore, we hypothesise that the rectum is the most sensitive area of the colon to 5-FU activity, which could be regarded as the area of major focus in future drug toxicity studies.

The case of TKIs

Tyrosine kinase inhibitors (TKIs) are low molecular weight pharmaceuticals whose main role is the selective inhibition of the activity of protein-tyrosine kinases [62]. These proteins are essential in modulating growth factor signals, thus they are highly associated with cancer development. When activated, they can promote proliferation and growth of tumour cells, anti-apoptotic effects, angiogenesis and metastasis. Furthermore, activation of protein kinases by somatic mutation is a recognized mechanism of tumorigenesis [63]. This explains why these protein kinases are important targets in cancer treatment. TKIs have proven to be effective and somewhat more efficient than classical pharmaceuticals in the treatment of diseases that develop from dysregulated kinase activity, such as cancer. Therefore, they are considered as the novel class of anticancer drugs, some of them already being used as targeted treatment of several malignancies, including advanced or metastatic GI stromal tumours, leukaemia or severe forms of non-small cell lung cancer (NSCLC) [63]. Since the first TKI drug (imantinib) got approved for cancer treatment, researchers have developed new compounds, managing to have seven more TKIs approved to date: dasatinib, gefitinib, lapatinib, erlotinib, sorafenib and sunitinib [63]. There are other TKIs still under investigation or in clinical studies. Despite their differences in the target protein kinase, pharmacokinetic profile and specific adverse effects, TKIs share the same mechanism of action, which is based on competitive inhibition of ATP at the catalytic binding site of the target enzyme [63]. A more detailed description about these TKIs and their main features can be found elsewhere [63,64].

A distinction can be made between receptor or non-receptor protein kinases, which are inhibited by different types of TKIs. Receptor tyrosine kinases are involved in the transduction of extracellular signals into the cytoplasm, which culminates in affected biological processes, whereas non-receptor ones are responsible for propagation of intracellular signals [63]. There are four main types of TKIs according to the type of tyrosine kinase they target, namely BCR-ABL tyrosine kinase and c-KIT inhibitors (imatinib), epidermal growth factor receptor (EGFR) TKIs (gefitinib, erlotinib, lapatinib and canertinib), vascular endothelial growth factor (VEGF) TKIs (semaxinib, vatalanib, sutent and sorafenib) and platelet-derived growth factor receptor (PDGFR) inhibitors (imatininb and leflunomide) [63]. Sunitibib is regarded as a multi-targeted TKI [64]. Mutations in those factors lead to the development of different types of cancers. For instance, mutation in the BCR-ABL tyrosine kinase is characteristic in patients with chronic myeloid leukaemia and acute lymphoblastic leukaemia, whereas mutation in EGFR domain is highly associated to non-small cell lung cancer [63,64].

Regarding adverse toxic effects, most TKIs have shown to cause anaemia, thrombopenia and neutropenia, as well as edema, hypothyroidism, nausea, vomiting and diarrhoea. Among the already approved cancer therapy compounds, those in which diarrhoea is severe and dose-limiting include gefitinib, lapatinib, erlotinib, sorafenib and sunitinib [64]. Patients taking erlotinib have shown the most aggravated intestinal damage due to possible perforation [64].

Despite the promise and success of TKIs in the treatment of several malignancies, the challenge related to the development of cancer resistance is still a problem to overcome. Furthermore, as TKIs are still a novel class of drugs, studies on their safety and toxic profile, efficacy, mechanism of action and resistance are needed to better understand how they function. This is important for their further improvement and application in clinical practice.

Studies on transcriptomic responses

So far, there is one study focusing on transcriptomic responses after treatment with erlotinib, combining and comparing results obtained from rat small intestine epithelial cell (IEC-6), CCD 84441 CoN human colon epithelial cells and mice [65]. This study aimed at specifically investigating erlotinib-induced toxicity of intestinal epithelium via

endoplasmic reticulum (ER) stress, which authors believe it might be a potential mechanism of action that contributes to the occurrence of diarrhoea in patients. Results showed that erlotinib increased the permeability of the intestine epithelial cells IEC-6 confirmed by downregulation of mRNA expression levels of *Cdh1* and *Itgb1*, after 12 h exposure. *Cdh1* and *Itgb1* code for E-cadherin and β1-integrin, respectively, both important junctions of intestine epithelium. The decrease in E-cadherin concentrations in a dose-dependent manner additionally confirmed those results. Furthermore, erlotinib induced an increase in the levels of pro-inflammatory cytokines, namely IL-6 and IL-17E, also confirmed by upregulation of mRNA levels of their respective genes *IL-6* and *IL-7E* in a dose-dependent manner. Suppression of growth and increased apoptosis was observed in a dose- and time-dependent manner as LDH levels were significantly increased. Around 36% of IEC-6 cells and more that 50% of CCD 84441 CoN cells were apoptotic after exposure. ER stress was evaluated in IEC-6 cells and confirmed as a side effect by the increase in some related protein levels (p-eIF2α, ATF4, XBP-1s and CHOP) [65]. Similar results were obtained after exposure of CCD 84441 CoN cells to the drug, demonstrating that ER stress plays an important role in intestine epithelium damage cause by erlotinib.

In the same study, occurrence of diarrhoea was evaluated as well by treating mice with erlotinib during 8 weeks [65]. After administration of several concentrations of the drug, diarrhoea occurred and the animals' body weight diminished in a dose-dependent manner. Inflammation processes and cells' disruption was evident, but more importantly, levels of E-cadherin were decreased and levels of CHOP were increased in the intestine epithelium. Likewise, levels of IL-6, IL4 and IL-17f were increased in a dose dependent manner, corroborating the results obtained *in vitro* [65]. Results of this study are promising and propose ER stress caused by erlotinib as a possible mechanism responsible for disruption of gut barrier integrity, apoptosis and inflammation of intestine cells, both *in vitro* and in mice.

Nevertheless, more studies on transcriptomic responses to TKI administration needed to clarify mechanisms involved.

Intestinal chronic toxicity caused by NSAIDs

NSAIDs are widely used in the treatment of several conditions such as rheumatoid arthritis, osteoarthritis, pain management (e.g. headaches, joint pain) and reduction of fever [66,67]. In addition to their analgesic and anti-inflammatory actions, NSAIDs are also used in the prevention of heart-diseases, myocardial infarction, strokes, thrombosis and Alzheimer [66,68]. More recently, NSAIDs have shown efficacy in preventing colorectal tumours and in the treatment of patients with familial adenomatous polyposis [66]. NSAIDs are taken daily by approximately 3 hundred million people, making it the most frequently used class of drugs worldwide, especially aspirin [67].

Due to the abundant use, the concern about toxicity caused by NSAIDs has grown as well. Indeed, NSAIDs have already been associated with several side effects, particularly concerning the intestines, in which they can lead to inflammatory intestinal disease or inflammatory bowel disease (IBD) [69]. Common adverse effects include ulceration and haemorrhagic lesions in the stomach and intestine, which increase the risk of perforation and bleeding. In the small intestine, ulceration and inflammation can lead to alterations in the mucosal permeability as well [68]. Despite the damage being subclinical in most cases, it can result eventually in more serious problems, including anaemia, obstruction and even death [68]. In the colon, previous colonoscopy analyses have shown the formation of colonic ulcers, diffuse colitis and major intestinal bleeding/perforation derived from NSAIDs abuse. Furthermore, such damage can lead to increased permeability of compounds (e.g. lactulose) and excretion of calprotectin in faeces, a biomarker of intestine inflammation. These risks are, however, dependent of the dose and type of NSAID, and they increase with individual's age or intestine condition (e.g. previous ulcerations).

There is not a clear mechanism by which NSAIDS lead to intestinal injury and toxicity since they can act via direct nonspecific irritation, impairment of mucosal barrier and epithelial reconstitution, local cyclooxygenase (COX) inhibition with suppression of prostaglandin (PGE) and other eicosanoids synthesis, reduction of blood flow to the mucosa, perturbations in neutrophil recruitment and intestinal flora [70]. Nevertheless, the main well-known mechanism of action of NSAIDs is the one that involves inhibition of COX enzyme, and consequently, inhibition of PGEs synthesis. PGEs, both PGE_1 and PGE_2, have a protective role of the intestinal mucosal lining through stimulation of gastric mucus and bicarbonate secretion, decrease in gastric acid production and increase of the intestinal mucosal blood flow. Therapeutic and/or adverse effects of NSAIDs derive from inhibition of such factors. PGEs production is controlled by COX-1 activity, one of the two COX enzyme isoforms that is constitutively expressed in several tissues including the intestines. On the other hand, COX-2 is usually absent in cells, being mainly induced at inflammation sites. Therefore, NSAIDs can be classified into 3 groups according to which of the COX isoforms they target: i. non-selective COX-inhibitors, which includes most NSAIDs (e.g. aspirin, ibuprofen, diclofenac and indomethacin), and exert effects in both COX-1 and 2, reversibly or irreversibly; ii. preferential COX-2 inhibitors (e.g. nimesulide); iii. specific COX-2 inhibitors (e.g. celecoxib and rofecoxib).

Non-selective COX-inhibitors have been more associated with intestinal damage and toxicity [71], as they lead to the inhibition of both the synthesis of cytoprotective and inflammatory PGEs, as well as potential platelet aggregation. Hence, inhibition of COX-2 by specific NSAIDs is thought to be preferable over COX-1 inhibitors due to reduced adverse effects and increased safety of treatments, being at least as efficient in handling pain, fever and inflammatory conditions. Nevertheless, some reports contradict this view as they describe intestinal injury induced by chronic use of COX-2 inhibitors with the same probability of non-selective COX-inhibitors NSAIDs [72–74], leading to the hypothesis that COX-2 might also have a role in maintaining the integrity intestine mucosa [71].

Non-selective COX-1/2 inhibitors, such as aspirin, are still among the most consumed NSAIDs, particularly by patients that are under medication for chronic pain or rheumatoid disorders, making them more prone to suffer from intestinal toxicity, which also aggravates with age [71]. Furthermore, clinicians are still reluctant to prescribe selective COX-2 inhibitors due to the likelihood of developing cardiovascular complications [71]. Therefore, it is important to completely understand the mechanism of intestinal – toxicity induced by these pharmaceuticals. Insight on the gene profile alterations can be useful to improve our knowledge in that matter and help predicting such adverse effects. Along with this, toxicity biomarkers specific to NSAIDs can also be discovered, which would result in better prediction of intestinal injury before the damage becomes too severe or irreversible.

In vitro studies

So far, we have found 4 studies in colon cell lines that targeted alterations in gene expression after exposure to NSAIDs. Germann et al. exposed the cell line CC531 derived from rat colon carcinoma to aspirin, which resulted in 121 genes being significantly altered after the exposure (80 genes upregulated and 41 downregulated). Among these genes, the effects of aspirin were more significantly noticeable in genes involved in WNT and RAS signaling pathways, namely *cyclin D1, cyclin E, c-myc, fosl1, c-fos, follistatin* and *Cd44* [75].

In another study, the effect of indomethacin, a non-selective COX inhibitor, and with two selective COX inhibitors NS-398 and SC-560 were tested in the IEC-6 rat small intestine epithelial cell line [70]. NS-398 and SC-560 are selective COX-2 and COX-1 inhibitors respectively, and they have only been used in *in vitro* experiments so far. This study aims at checking for alterations in mRNA and protein expression for calpains 8, 2 and 1, and calpastatin [70]. Calpains are cysteine proteases involved in numerous cellular processes including cell migration and invasion. It was observed that indomethacin and NS-398 caused a

decrease in the expression of the three calpains. SC-560 exposure led to the decrease of calpains 8 and 2, but the opposite was observed in calpain 1, whose expression increased. Concerning calpastatin expression, it was not significantly affected by any of the exposures [70].

In vitro exposure to celecoxib was performed in two other studies [76,77]. Deng et al. studied the effect of celecoxib in CD133 expression using two human colon cancer cells (HT29 and DLD1) [76]. After exposure, a significant decrease was observed in CD133 expression levels with the increase in concentration and duration of exposure in HT29 cells. CD133 mRNA relative expression was also found decreased after drug exposure in both cell lines. Moreover, stemness genes (*CEACAM5, CDK1A, STAT2, GDF15, ADFP, ICAM1, CEACAM6, APOL2, TUBE1, STAT1, UBD, VLDLR, LIF* and *CXCL2*) were found downregulated and differentiation genes (*LGR5, GABRP, E2F8, CDK2, POU5F1P1, ABCB1, FGFR4, CXCR4, PROM1, WNT11, MYB, EDN1, PROM2 and CCNE2*) were found upregulated [76]. In a more recent study with celecoxib, the cell line HT29 was also used in order to evaluate drug effects on cell viability and in the expression of COX-2, Caspase-3, VEGF and NF-kB genes [77]. However, the results showed that celecoxib did not significantly affect cell viability and caspase-3 (indicator of apoptosis). Only the expression of *COX-2* was affected, being found significantly decreased after exposure [77].

Animal model studies

Only one *in vivo* study was performed to evaluate the whole genome transcriptomic responses associated with NSAIDs intestine toxicity so far [78]. In this study, Ryu et al. compared 2 animal models (zebrafish and rat) with the human colonic adenocarcinoma cell line Caco-2, aiming at developing an alternative animal model (zebrafish) to study intestinal toxicity induced by drugs. All three models were exposed to indomethacin, diclofenac and methotrexate [78]. Specific biomarkers were selected to evaluate the effects of those drugs, including biomarkers of metabolism, oxidative stress, apoptosis, inflammation and intestinal structural changes, and ultimately compare the results between the different models. Overall, the majority of the selected genes were upregulated in the three toxicity models after exposure to one of the three compounds, namely *CYP3A* (metabolism); *iNOS, Hmox1, Sod1* and *Gpx1* (oxidative stress); *Bax, Bcl-2, Casp9* and *p53* (apoptosis); *IL-1β, TNF-α* and *Tlr2* (inflammation). Expression levels of NF-kB and Occludin (intestinal tight junction) were decreased after exposure to the drugs [78]. Altogether, this study shows putative genes that are affected by NSAIDs exposure that can be part of the transcriptomic signature of these NSAIDs.

Studies on human samples

Celecoxib, indomethacin and aspirin are the NSAIDs that are mostly studied in humans, despite the number of studies being rather limited. Glebov et al. included 25 pre- and post-treatment colon biopsies from which 14 patients took orally 200 mg of celecoxib and 11 patients took 400 mg of the same drug, twice a day during 12 months. Moreover, 14 colon biopsies from patients treated with placebo were also included with the aim to determine gene expression profiles at baseline and after treatment, in normal colonic mucosa [79]. It was observed that 173 genes were significantly differentially expressed between pre- and post-treatment biopsies with celecoxib. Furthermore, 75% of the genes presented with greater fold differences in patients treated with the highest dose of celecoxib, demonstrating dose-dependent changes in gene expression. The most significantly altered genes between pre- and post-treatment samples are involved in immune and inflammatory responses, angiogenesis, cell adhesion and proliferation, transforming growth factor-β signalling pathway and apoptosis [79].

In another study, indomethacin was evaluated in order to assess the effect of the drug in the expression of genes in the duodenal mucosa of healthy subjects [80]. Duodenum samples were collected from 16 patients who took either placebo or indomethacin (125 mg) to evaluate transcriptomic responses with and without treatment, in normal duodenal mucosa. Results showed that 7 genes were differentially expressed between placebo and indomethacin groups, among which three genes were downregulated in samples exposed to indomethacin (*CYP1A1, G6PC* and *NFIL3*) and four were upregulated (*MMP3, CIART, PER3* and *GPRC5A*) [80]. In turn, Thomas et al. conducted a study with aspirin including colon samples from 44 healthy men and women who were given placebo and aspirin in different periods. The aim of the study was to evaluate the effect of a 60-day aspirin supplementation on normal colon epithelial and stromal gene expression as well as determine changes in prostaglandin 2 (PGE2) levels to check for tissue specific alterations after exposure to the drug [60]. However, no significant differences in gene expression were found in response to aspirin. Concerning tissue PGE2 levels, they were found decreased with aspirin compared to placebo. Notably, authors did find transcripts differentially expressed between colon epithelium and stroma, demonstrating the importance of tissue-specific gene profiling in future studies.

Lastly, Slaterry et al. performed a study to evaluate transcriptomic responses in colon tissue that could be related to diet, lifestyle and oxidative stress [81]. Colonic non-tumour tissue was collected from 53 colon cancer patients (female and male) that were known to have used aspirin and/or ibuprofen. Total RNA sequencing was performed; however only 3 genes were found significantly differentially expressed between non-exposed and exposed to NSAIDs. These include *TMCO6* and *STEAP3,* both upregulated, and *ST8SIA4*, downregulated [81]. The first one (*TMCO6*) is associated with protein transporter activity and intracellular protein transport. The second gene (*STEAP3*) is involved in metal ion binding, apoptotic processes, cell cycle and TP53 signalling pathway, whereas *ST8SIA4* is associated with cellular protein metabolic processes, nervous system development protein linked to glycosylation.

The availability of GI-toxicity data on NSAIDS and particularly transcriptomic data is even more limited than of 5-FU. This automatically implies a lack of information on the mechanisms underlying those effects. Generating such data would allow developing predictive methods that could be used in the early phases of drug development. This requires studies on gene profiles and other molecular markers in association with intestinal toxicity induced by NSAIDs.

Concluding remarks and future perspectives

Overall, the number of studies investigating gene expression profiles associated with drug induced intestinal toxicity, either *in vitro*, animal models or human subjects, is quite limited. Nevertheless, it is possible to infer a few possible candidate biomarkers for drug-induced intestinal toxicity based on some of the results obtained in such studies. Regarding 5-FU, possible biomarkers could be the increased expression of the genes *SSAT* and *MAT-8*, the polymorphic genes *DPYD*, *TYMS* and *MTHFR,* combined with increased levels of citrulline and cytokines including IL-6, TNF-α and IL-1β, and decrease of DAO levels. TKIs have shown to cause a decrease in mRNA expression levels of *Cdh1* and *Itgb1*, and consequently, decrease in the levels of E-cadherin and β1-integrin, respectively, as well as the increase in the levels of LDH, CHOP and the cytokines IL-6, IL-17E and IL-4. Finally, NSAIDs data was not very concordant, but increased expression of genes such as *CYP3A, Hmox1, Gpx1, Casp9, TMCO6, STEAP3* and decreased expression of *ST8SIA4* was reported. Likewise, increased levels of IL-1β and TNF-α, and decreased levels of PGE_2 were reported.

Although evidence points out that several different molecular events are occurring depending on the drug and its mechanism of action, the same effects are observed at the cellular level. Therefore, it seems that different pathways of drug-induced toxicity, such as inhibition of TS by 5-FU, increase of ER stress by TKIs or NSAIDs impact on the genes related to stem cell-like properties, culminate in intestinal toxicity. Despite these insights, more robust and drug-specific studies are still needed to better understand the mechanism underlying intestinal toxicity and how it develops in order to generate a general panel of

predictive biomarkers of GI-toxicity.

The majority of the studies investigating drug-induced responses in intestinal cells have been performed *in vitro* using colon cancer cells. The translatability of *in vitro* to human *in vivo* situation is quite challenging, and using cancer-derived cells to gain insight of what happens in healthy human cells is even more complicated. Although animal models take pharmacokinetic effects into account that cannot be studied *in vitro*, they may not correctly reflect such responses in humans. This is clearly supported by attrition rates in clinical trials demonstrating that rodent-derived data poorly translate into human risks. On the other hand, human studies performed so far are also limited in the sense that they include colon cancer patients or therapies with a combination of drugs rather than monotherapy with the target drug. Therefore, investigation of transcriptomic responses regarding drug-induced intestinal toxicity is still very limited, compared to other organs, hampering the generation of models of intestinal toxicity.

As mentioned previously, *in vitro* and rodent data poorly correlate with human risks during clinical trials. In an attempt to overcome this setback, as well as reducing animal experiments, *in vitro* culture systems have undergone great development from the traditional 2-dimensional (2D) to 3D culture models of human organoids, which seem to a more promising alternative to rodents to investigate biomarkers. Toshiro Sato et al. was the first investigator who was able to develop intestinal organoids from stem cells of the small intestine crypts, being the first major technological advance in 3D culture [82]. Due to its remarkable work it is now possible to perform long-term culture of organotypic 3D systems of any human tissue or organ [83–85].

Organoids have demonstrated to present features quite similar to *in vivo* tissue or organs, in terms of proliferation, differentiation and behaviour [86]. Therefore, they are regarded as the future tool to study biological processes from cellular differentiation to homeostasis and development of diseases [84,85]. Organoids have also shown potential for high-throughput screening for drugs efficacy and/or toxicity and to investigate different gene expression or signalling pathways that differ between normal and abnormal conditions [85]. Intestinal organoids, either derived from the small intestine or colon, are no exception as they can be used to gain new insights into intestinal development, homeostasis, diseases, development of personalized therapies, evaluation of gene expression profiles, and evaluation of intestinal toxicity caused by certain drugs [22,84]. In addition, the improvement of the mechanistic understanding of drug-induced toxicity in both organs provided by this culture technique will provide better and more tissue-specific biomarkers, and potentially enable the discrimination between small intestine and colon toxicity.

The development of intestinal organoid models as an alternative to animal studies for the prediction of *in vivo* intestinal toxicity is definitely a huge step forwards in the development of safe medicines. Furthermore, the promise of an improved translatability between these *in vitro* model systems and humans is a major advancement in the understanding of relevant pathways of drug toxicity and for providing a quantitative toxicology model for future drug investigations. These advances are the focus of the TransQST (Translational Quantitative Systems Toxicology) project, which aims to provide innovative methodologies and tools for systems toxicology modelling and translational toxicity biomarkers derived from the recent *in vitro* culture models [87] (http://transqst.org/).

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Declaration of Competing Interest

We wish to confirm that there are no conflicts of interest to declare associated with this publication.

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