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Review article

In vitro models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review

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ABSTRACT

Absorption, distribution, metabolism and excretion (ADME) studies represent a fundamental step in the early stages of drug discovery. In particular, the absorption of orally administered drugs, which occurs at the intestinal level, has gained attention since poor oral bioavailability often led to failures for new drug approval.

In this context, several *in vitro* preclinical models have been recently developed and optimized to better resemble human physiology in the lab and serve as an animal alternative to accomplish the 3Rs principles. However, numerous models are ineffective in recapitulating the key features of the human small intestine epithelium and lack of prediction potential for drug absorption and metabolism during the preclinical stage.

In this review, we provide an overview of *in vitro* models aimed at mimicking the intestinal barrier for pharmaceutical screening. After briefly describing how the human small intestine works, we present i) conventional 2D synthetic and cell-based systems, ii) 3D models replicating the main features of the intestinal architecture, iii) micro-physiological systems (MPSs) reproducing the dynamic stimuli to which cells are exposed in the native microenvironment. In this review, we will highlight the benefits and drawbacks of the leading intestinal models used for drug absorption and metabolism studies.

1. Introduction

Absorption, distribution, metabolism and excretion (ADME) are biological processes that involve several organs (i.e. intestine, liver, kidneys), finally determining the drug levels in tissues [1]. Presently, poor pharmacokinetic (PK) and pharmacodynamic (PD) properties of drug candidates have been identified as potential causes for high rates of drug failures, since only one compound of hundreds is normally approved by the Food and Drug Administration (FDA) [2]. For this reason, it is increasingly evident that ADME profiling represents a fundamental step in the selection of a new chemical entity (NCE). As a consequence, the assessment of ADME mechanisms became an essential part of the drug discovery pipeline, starting from the hit identification stage to lead generation and optimization, and the early pre-clinical phase of drug development [3–6]. Oral ingested compounds, which are the most commonly administered to patients, are subject to all ADME processes; in particular, the absorption, mainly occurring at the intestinal level, is the first crucial step establishing the drug fraction which successfully enters the blood circulation. It is thus fundamental to develop drugs that can efficiently penetrate the intestinal epithelium. Here, multiple cells, carriers and enzymes are involved in the transport and metabolism of the delivered substances [4]. In fact, the gastrointestinal tract (GIT) is one of the most active and dynamic organs in the body representing the first physical and chemical barrier encountered by exogenous compounds that come from the oral cavity [7]. The GIT anatomy and physiology (e.g. pH environment) together with the drug physio-chemical properties as well as the dosage form type (e.g. capsule, solution, emulsion, tablet) condition the oral absorption of natural and artificial compounds [8,9], finally affecting their bioavailability. Recent reports highlight that 90% of the orally taken drugs have poor bioavailability as a consequence of inefficient intestinal absorption, thus hampering their ultimate therapeutic efficacy [10,11].

Basically, oral bioavailability (F) refers to the dose that reaches intact the systemic circulation. It is defined as:

$$F = Fa*Fg*Fh \quad (1)$$

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where F_a is the fraction absorbed, i.e. part of the dose absorbed into the enterocytes of the intestinal membrane; F_g is the intestinal bioavailability, i.e. part of the dose escaping the metabolism in enterocytes; F_h is the hepatic bioavailability, i.e. part of dose entering the liver that escapes the hepatic metabolism [12].

The raising issue of low oral bioavailability led to increase drug doses, resulting in high toxicity and complication risks, as well as economical wastes, or even the change of the route of administration [13]. Hence, it is mandatory to clarify and predict the complex biological phenomena taking place from the mouth to the intestine, to properly conceive and design new orally delivered therapeutics.

Nowadays, animal testing still represents a gold standard in preclinics as it is capable to reproduce ADME processes mediated by systemic blood circulation [14]. Despite this fact, it is difficult to discern and decouple all the parameters involved in the passage of a molecule across the several biological barriers encountered in vivo [15]. Furthermore, animal-based assays often fail in predicting drug absorption and first-pass metabolism in humans due to species differences in transporters and enzymes expression as well as immune responses [16,17]. Moreover, *in vivo* models are generally labor-intensive, expensive and time-consuming. Therefore, there is a need to develop and promote alternative *in vitro* platforms in pharmaceutical studies in agreement with the 3Rs principles (Reduce, Refine, Replace) [18,19].

Conventional cell monolayers are considered the leading models for evaluating the ADME effects of orally ingested drugs. These flat systems are simple, cost-effective and standardized tools for replicating biological mechanisms *in vitro*. However, 2D cultures cannot precisely reproduce the physiological environment of human tissues, since cells adhere to a planar surface and display incomplete or altered cell-cell and cell-matrix interactions [20]. Cells grown on plastics are over-exposed to chemicals added in the culture medium; this frequently generates false-positive results for a drug candidate thus impairing the clinical translation of NCEs [21–23].

As a result, 3D cell culture systems have been realized to better recreate native tissue functionalities and pave the way for more predictive *in vitro* drug testing platforms [24]. 3D models provide a more physiologically relevant niche for cell growth if compared with 2D cultures, still maintaining their benefits over animals. Consequently, when drugs are tested in a 2D or 3D context, their effectiveness drastically changes [25]; specifically, cells cultured in a 3D setting are often more resistant to pharmaceutical treatments than cells grown as a monolayer, similarly to what happens in the human body [22,25]. Nevertheless, despite the great improvements, 3D *in vitro* models (e.g. organoids, spheroids etc.) lack of organ-specific dynamic stimuli significantly affecting cells behavior. The study of ADME mechanisms particularly requires the presence of fluid flows resembling the bloodstream, and thus the drug systemic circulation and its transport kinetics.

To address this need, researchers have recently fabricated microphysiological systems (MPS) by integrating such 3D cell cultures with microfabrication techniques to impose dynamic culture conditions at the smallest biologically acceptable scale [26]. Such MPS allow to set controlled fluid flow patterns, provide mechanical forces and an efficient transport of substances as well [27]. Especially, organs-on-chips (OOCs) support the culture of substructures considerable as microscaled functional units of living organs under highly reliable conditions [28]. So far, these technologies offered the chance to recreate models of kidney [29], liver [30], brain [31], heart [32], skeletal muscle [33] and intestine [34] with a significantly reduced consumption of costly reagents, number of cells and drugs [35,36]. The use of MPS enhanced the automatization and parallelization of experiments for the collection of large-scale data sets of ADME profiles, potentially speeding up the drug discovery and development processes [24,28]. However, the over-miniaturization of these models in some cases may affect the faithful reproduction of organ-level functions, requiring an experimental complexity that simultaneously hinders the practical operation and manipulation of the system [23,36,37].

Hence, this review details the intestinal models currently adopted for investigating the absorption of orally administered compounds that include 2D conventional intestinal monolayers, complex 3D tissues, and novel emerging physiologically relevant MPS culture systems.

In short, we will focus on intestine models that reproduce the multiple features of the human organ and accurately predict the time-dependent drug absorption profiles occurring *in vivo*.

2. Physiology of the GIT

Drugs orally delivered follow a precise path starting from the mouth, passing through the esophagus and the stomach, terminating the travel in the large intestine. This alimentary canal is lined with a mucosa, which is composed of an epithelium covering a lamina propria where blood and lymphatic vessels reside. After traversing the first portions of the GIT, a drug (e.g. tablet) enters the stomach, where it encounters an acidic environment (pH ranges from 1.5 to 3.5 [38]) and specific enzymes involved in digesting food [39]. Muscular contractions also occur to break up the solid tablet into smaller particles, resulting in its disintegration and dissolution. In particular, the drug aqueous solubility, lipophilicity, and hydrophilicity, as well as the presence of excipients, are crucial factors in determining the successful fulfilment of these preliminary absorption phases [12].

Subsequently, the gastrointestinal content navigates to the small intestine (duodenum, jejunum, and ileum), which represents the major site of absorption of nutrients, water, electrolytes and xenobiotics [40]. Here, the absorptive mucosa is characterized by a simple columnar epithelium surmounting a highly blood perfused lamina propria, which maximize the possibility of molecules entering the systemic circulation through mesenteric vessels. Herein, the mucosa possesses a large surface area (250 m^2) with wide folds, villi and microvilli (Fig. 1A) which significantly impact the uptake rate by augmenting the available absorptive surface area [41]. Layers of smooth muscle are also present within the lamina propria inducing the villi to undulate and the luminal content to dynamically mix, thus promoting the ingestion of the substances [42].

The intestinal epithelium consists of a single layer comprising several types of cells, as shown in Fig. 1B. Specifically, enterocytes are the intestine predominant cells. They are columnar-type cells held together by tight junctions, which play a pivotal role in regulating the diffusion of small compounds and the exclusion of toxic large molecules. In addition, they are characterized by the presence of about 3000-7000 microvilli over their apical membranes, which greatly benefit the absorption mechanism [43]. However, enterocytes contain also a large number of drugs- and food- metabolizing enzymes for the xenobiotics and nutrients digestion as well as for the exogenous substance protection [44–46]. Therefore, enzyme-guided reactions taking place in the small intestine limit the oral bioavailability of administered compounds [47]. For these reasons, taking into account intestinal metabolism is thus necessary.

The enterocytic monolayer is interrupted by the residence of both enteroendocrine cells and Goblet cells. The firsts are responsible for releasing peptide hormones, which are correlated with tissue repair, angiogenesis, enterocytes differentiation and polarization [48]. Conversely, goblet cells produce mucus, a rheological substance covering the intestinal epithelium, which acts as a protective layer against noxious substances and microbial infections like bacteria or toxins. Importantly, this gel is the first barrier that filters and thus narrows the absorption of molecules, being essential in the maintenance of intestinal homeostasis [49]. Mucus properties and thus function vary along with the GIT; for example, the small intestinal mucus contains large pores up to 2 μm^2 for ensuring a massive absorption of nutrients [50]. Recently, researches demonstrated that alterations in the mucus architecture and content may lead to several pathologies, confirming the remarkable impact of this viscous gel on intestine health [51].

Columnar intestinal stem cells (ISCs), located in the so-called crypt base, are capable to differentiate into all other intestinal cell types,

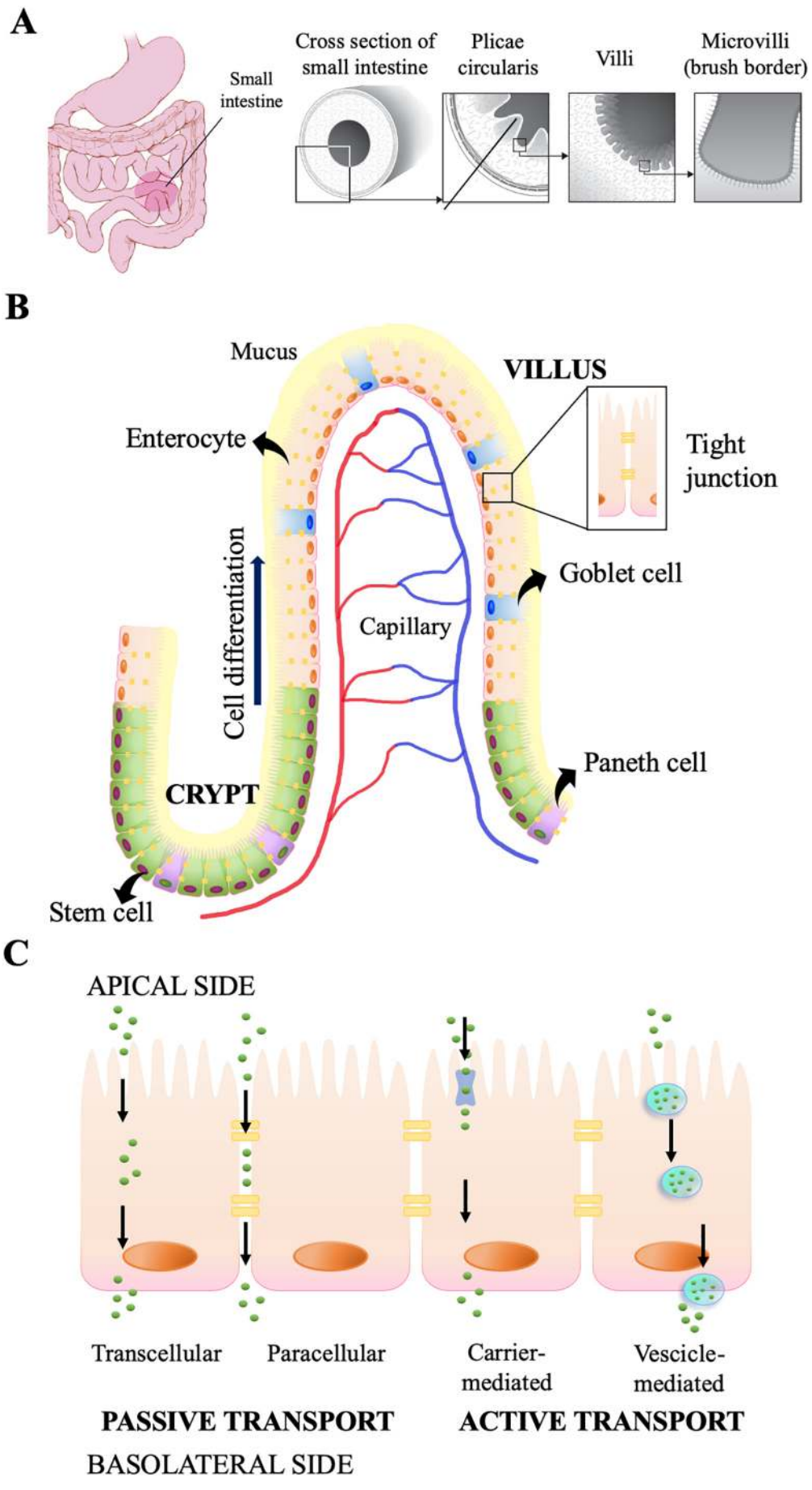

Fig. 1. A cartoon depicting the architecture of the small intestine and its transport mechanisms. (A) GIT consists of the esophagus, stomach, small intestine, large intestine, and rectum (left, [62]). Schematic cross-sectional views of the small intestine tissue with an increasing magnification (right). Wide folds of mucosa (plicae circulares) are characterized by finger-like villi projections protruding into the intestinal lumen; microvilli are also present over enterocytes to increase the absorptive area; reprinted and adapted from [63], Copyright (2008) with permission from Elsevier. (B) Illustration of a typical basal crypt/villus units of the small intestinal mucosa. This columnar epithelium comprises several types of adjacent cells, which are firmly joined by tight junctions; a layer of mucus surmounts the apical cell membranes providing an additional physical and biochemical barrier, while blood vessels highly perfuse the tissue maximizing the possibility of molecules to enter the blood circulation. (C) Transport pathways of substances across the intestinal epithelium; absorption can occur in parallel involving passive (transcellular and paracellular) and active (transcellular carrier-mediated and vesicle-mediated) mechanisms.

providing an extremely high intestinal self-renewal preserving the tissue integrity and function [52,53]. Finally, also Paneth cells reside in the crypt region and are specialized in supporting ISCs within the niche by secreting growth factors and producing antimicrobial peptides [54,55].

Ingested drugs travel from the luminal side of the intestinal epithelium to the basolateral one to enter the mesenteric vessel through various mechanisms involving either passive or active transports (Fig. 1C).

Passive transport may occur via the transcellular pathway, in which drugs pass through the cell membrane, and the paracellular pathway, in which drugs pass through the intercellular spaces. Both of them are regulated by Fick's law of diffusion:

$$J = -D\,dC/dx \quad (2)$$

where J is the rate of transfer per unit area (flux) ($g/cm^2/h$), dC is the concentration gradient (g/cm^3), dx is the linear distance travelled (cm) and D is the diffusion coefficient (cm^2/h).

Besides this, the apparent permeability coefficient (P_{app}) describes the amount of drug crossing the barrier per unit time and unit area (A), and it can be calculated in either the apical to basolateral (influx) or the basolateral to apical (efflux) direction through the equation below derived by Fick's law:

$$P_{app} = \frac{dQ/dt}{A\,C_0} \quad (3)$$

where dQ/dt expresses the rate of drug appearance in the acceptor side and C_0 is the initial drug concentration in the donor side [56]. Notably, P_{app} value is usually adopted to determine the oral fraction absorbed (F_a) in humans [57,58].

The paracellular mechanism is the primary diffusion process for hydrophilic substances, occurring through the water-filled intercellular spaces (tight junctions). Hence, the paracellular passage is strictly referred to the integrity of the barrier, which can be monitored *in vitro* by measuring the trans-epithelial electrical resistance (TEER) [59,60].

On the other hand, transcellular transport mostly guides lipophilic compound uptake across the lipid cell membranes.

Nonetheless, also active transport promotes the passage of compounds towards the basolateral side of the small intestine. Transcellular carrier-mediated mechanisms and the vesicular-mediated endo- or trans-cytosis actively carry drugs [61]. In addition, efflux transporters present on the apical or basolateral side can modulate the transfer from the cell cytoplasm back into the intestinal lumen, decreasing the net amount absorbed.

Accordingly, complex intestinal epithelium models recapitulating these various cell dynamics are required for carrying out more predictable pharmacological investigations *in vitro*.

3. 2D *in vitro* models to evaluate intestinal absorption

Nowadays, 2D culture systems remain widely employed to determine the bioavailability of an orally delivered drug, mainly because of their low costs, high reproducibility, and ease of manipulation.

In particular, 2D *in vitro* models conventionally employed in pharmacology can be divided into two main categories: (i) synthetics models based on lipidic membranes, which offer a great reproducibility and stability, used to study passive diffusion processes [64], and (ii) cell-based cultures that are living and more reliable systems allowing broader spectrum analyses of intestinal absorption (Fig. 2A).

3.1. Synthetic models

3.1.1. PAMPA

The Parallel Artificial Membrane Permeability Assay (PAMPA) is a cell-free permeation system that reproduces *in vitro* the phospholipid composition of the desired human biological barrier (e.g. gut) for evaluating the passive transcellular absorption [65]. Especially, this completely artificial technology consists of two compartments separated by a membrane soaked with a mixture of phospholipids dissolved in an organic solvent, mimicking the lipidic membrane of the enterocytes [66].

Considering that 80–95% of commercial drugs are primarily absorbed through passive diffusion, PAMPA represents a useful tool for an early-stage ADME screening of orally administered compounds [66,67]. Particularly, the rate of permeation across the lipid-based membrane represents a valid indicator of a drug absorption potential. In fact, several studies demonstrated a good relationship between the permeability data measured by PAMPA and those obtained with the Caco-2 cell-based model for drugs transcellularly transported [68].

On the other hand, PAMPA is unable to classify hydrophilic compounds transferred via paracellular pathways. To overcome this limitation, other variants of the original structure were developed by changing the key factors influencing the membrane performances (e.g. the nature of the filter support, pH conditions and lipid membrane composition) [7]. Specifically, the Double Sink PAMPA showed a stronger correlation with the human data and an improved predictive potential for poorly water-soluble drugs [69].

Hence, PAMPAs revealed to be rapid, solid and low-cost platforms for measuring permeabilities of lipophilic molecules across several biological barriers (gut but also skin, blood-brain barrier), consequently limiting cellular-based assays or animal testing in the pre-clinical scenario [70,71]. Nevertheless, their potency and applicability are hampered by their acellularity and the presence of organic solvents, which can interact with the supportive filter [64]; thus other synthetic alternatives have been recently developed [7].

3.1.2. PVPA

The Phospholipid Vesicle-based Permeation Assay (PVPA) is another artificial membrane-based system currently employed for the pharmaceutical characterization of NCEs [72]. As PAMPA, PVPA was introduced for *in vitro* replicating the lipid composition of biological epithelia to measure the permeability of passively delivered drugs [73,74].

Unlike PAMPAs, this is a synthetic organic solvent-free platform consisting of a tight barrier fabricated by depositing liposomes between the pores and on the top of filter support emulating the phospholipid bilayer, such as that of the intestinal cell membranes [75]. Typically, PVPA is capable to better envision the human absorbed fraction compared to PAMPA by performing experiments over a more biologically-relevant context, providing more similar results to Caco-2 cells cultures [64,73,74].

Over the years, diverse advancements of the PVPA led to remarkably enhance the biomimetism of this model. For instance, a negative charge was set up, and the lipid composition of the membrane was modified to perfectly match the composition of the intestinal barrier, thus allowing to carry out permeability tests under physiologically relevant pH conditions. In this way, an accurate capability to correctly classify at least 80% of tested compounds was exhibited accordingly to the *in vivo* absorbed fractions [74,76].

PVPAs is considered as an established approach for precisely assessing *in vitro* the absorptive potential of lipophilic drug candidates with high reproducibility and greater handling compared to cell-based models [74,76]. Nonetheless, significant morphological and physiological features are not expressed, such as the presence of villi and active transport proteins, differently from cell-based assays (Table 1). Consequently, PVPA, as well as PAMPA, remains a suitable tool only to predict the permeability of drugs transcellularly absorbed.

3.2. Cellular models

3.2.1. CACO-2 cell line

The Caco-2 cell line, isolated for the first time by Fogh *et al.* [77] from a human colon adenocarcinoma, is the most widely used cell-based model to *in vitro* simulate the physical and biochemical barrier of the

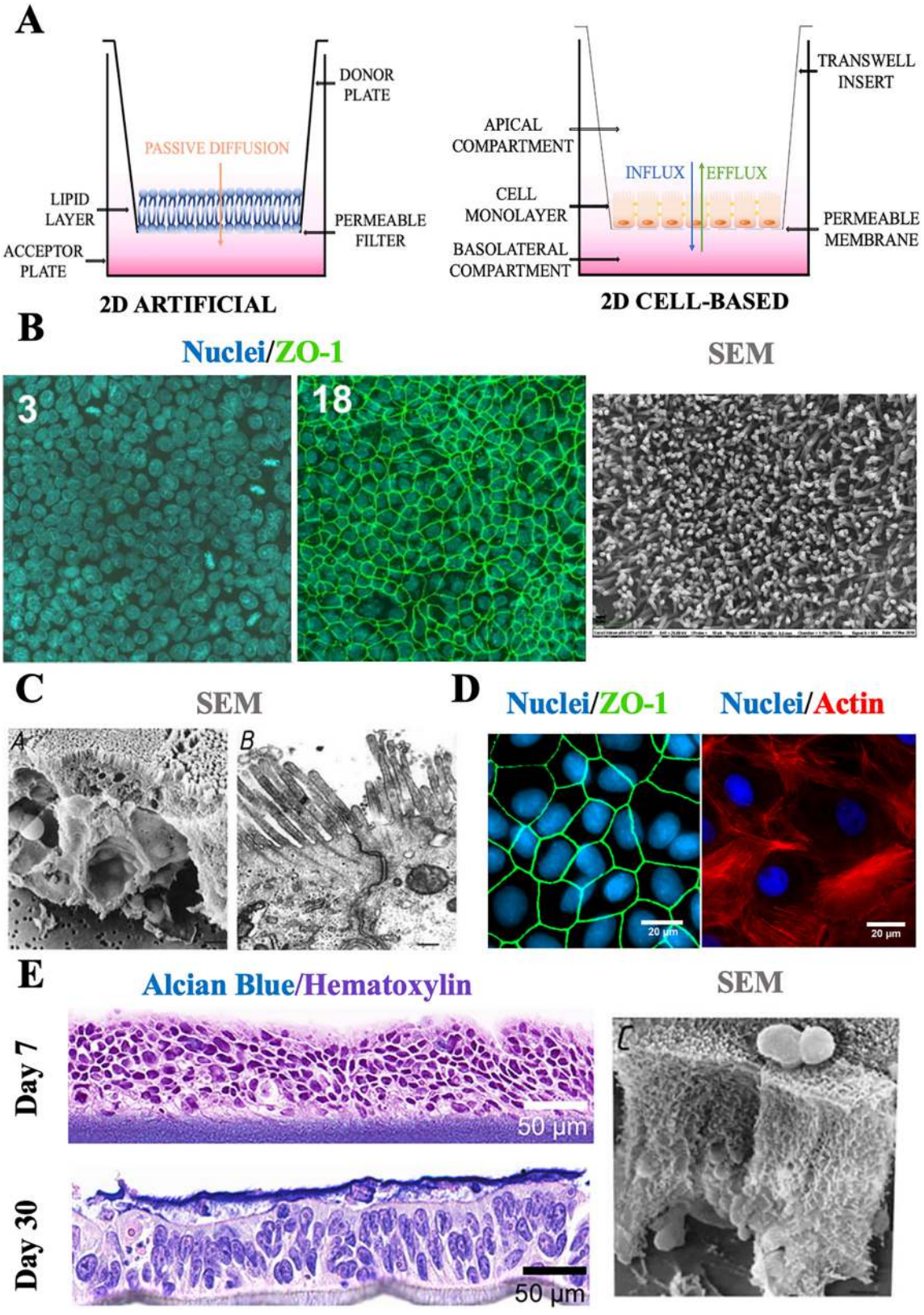

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Fig. 2. 2D culture systems currently used to determine the bioavailability of orally delivered drugs. (A) Scheme of artificial lipid-based (left) and cell-based (right) monolayers setup. (B) Development of tight-junctions in 2D Caco-2 cell monolayers after 3 (left) and 18 (middle) days of plating. Pictures are obtained by merging 100-frames z-stacks of the junction-mediating ZO-1 protein; reprinted and adapted from [166], Copyright (2012), with permission from Elsevier. SEM image showing a highly packed microvilli pattern over Caco-2 cells after 21 days of culture (right, [167]). (C) A side view of TC-7 cells cultured on a polycarbonate filter with brush borders and microvilli (left, SEM image); scale bar = 1.9 μm; two adjacent TC-7 cells displaying microvilli and strictly held by a tight junction (right, TEM image); scale bar = 0.4 μm; reprinted and adapted from [134] Copyright (2001), with permission from Elsevier. (D) Fluorescence images demonstrating the establishment of tight junctions (left) and a dense structure of actin filaments (right) in a 2D MDCK cell-based culture [168]. (E) Histology of an HT29-MTX cell monolayer; cells are organized in a multi-layered structure after 7 days, whereas they are well polarized and covered with mucus (stained in blue) after 30 days of static culture (left); reprinted and adapted with permission from [169] Copyright (2018) American Chemical Society. SEM image showing a lateral view of an HT29-MTX monolayer cultured on a polycarbonate filter with few microvilli and mucus droplets covering the apical membrane of the cells (right); scale bar = 2.4 μm; reprinted and adapted from [134] Copyright (2001), with permission from Elsevier.

human intestine in the pharmaceutical and nutraceutical field.

Caco-2 cells represent a gold standard accepted by regulatory authorities for ADME-Tox studies since they demonstrated to be particularly useful to (i) elucidate the absorption and metabolism of substances across the intestinal epithelium, (ii) predict the absorbed fraction in humans [78], and (iii) study, select and classify drug candidates under controlled conditions, according to the Biopharmaceutics Classification System (BCS) [79]. Indeed, despite their colonic origin, Caco-2 monolayers resemble most of the morphological and functional characteristics of the small bowel [80].

Conventionally, these cells display undifferentiated characteristics at the early stages of culture (after 3 or 4 days), whereas they are capable to reach an enterocytic phenotype at the late confluence (in about 3 weeks), expressing typical cytoskeleton proteins (e.g. villin [81]) and forming a polarized layer with brush-border microvilli (confirmed by sucrase immunoreactivity [82]) as well as tight junctions (confirmed by occludin antibodies staining [83]) between adjacent cells, as shown in Fig. 2B. However, these strict junctions entail a higher transmembrane resistance (150-400 $\Omega{\cdot}cm^2$[84]) respect the small intestine (12-120 $\Omega{\cdot}cm^2$[85]). This can be due to a smaller number and size of water-filled pores present in Caco-2 cells (about 3.7 Å) than the first intestinal tracts (8-13 Å) [59,86–88]. Accordingly, multiple studies, which investigated the paracellular uptake of hydrophilic compounds (e.g. atenolol, mannitol), indicated the inadequacy of this model in predicting the paracellular transport [89–91]. In fact, in literature, a systematic underestimation of the paracellular diffusion kinetics is extensively reported for Caco-2 cells. For example, remarkable divergencies respect to human jejunum regarding slowly and incompletely absorbed drugs were detected. The transport rates resulted to be 30- to 80-fold lower in the Caco-2 model, highlighting considerable limitations of such cell line in precisely replicating the physiological and anatomical jejunum properties [90]. Moreover, it is well-known that poorly absorbable drugs, which are unable to cross the villus narrow junctions, stay for a long time in the lumen, precipitating towards the crypt regions, where they can pass through the leakier crypt junctions. Nevertheless, this phenomenon cannot occur in flat monolayers because of the lack of a 3D crypt-villus structure [92]. Integrity alterations of the junctions by calcium-chelating solutions (e.g. EDTA) can be helpful to improve the permeability and thus the predictability of the paracellular pathway; however, the employment of these solvents is contraindicated [58].

Contrarily, the high similarity with absorptive enterocytes led Caco-2 monolayers to be considered an efficient intestinal membrane-mimicking model for studying the passive transcellular penetration of lipophilic compounds (e.g. propranolol and metoprolol). The permeability of rapidly and completely absorbed therapeutics differed 2- to 4-fold with respect to human intestine values for this route [90] and a high correlation was observed between Caco-2 P_{app} and *in vivo* F_a values. Surprisingly, reasonable correlations were also found for poorly absorbable compounds, fitting well human published PK data of 30 marketed compounds [58] and peptidomimetics [93,94]. Therefore, Caco-2 can be considered a valuable tool to screen passively transported compounds (correlation coefficient >83% [95]) as much as more complex models like *in situ* perfusion ones [96,97].

Less attractive results were obtained by using these cells to model active transport. Although some analyses provided promising outcomes about ATP-dependent paths [98] and gene profiles of intestinal transporters, such as P-glycoproteins (P-gps), involved in maintaining the intestine homeostasis by mediating xenobiotics efflux and secretion [99], expression patterns of other proteins (e.g. breast cancer resistant proteins (BCRPs) and multidrug-resistant proteins (MRPs)) [100–102] were observed to be considerably different from duodenal [95] and jejunal tissues [103]. For these reasons, even though FDA and European Medicines Agency (EMA) acknowledge this cancerous line as a surrogate for *in vitro* permeation assays, there is still an ongoing debate on whether it represents a biorelevant model for certain applications, such as the active absorption or the transporter-mediated drug-drug interactions [103]. Moreover, an intrinsic variability between and within laboratories as well as a strong dependence on culture time was noticed in the transporters content [103].

Likewise, some defects can be observed for metabolizing enzymes responsible for the elimination of many drugs (e.g. cytochrome P450 -CYP- isoforms) [44,45]. In particular, the deficiency of CYP3A4 enzymes represents a remarkable drawback to investigate Phase I metabolism and the first-pass effect of orally taken drugs [104,105].

To solve this, some groups tried to induce CYP3A4 expression by treating Caco-2 cells with 1-α-2,5-dihydroxyvitamin D3 or incorporating CYP3A4 with the NADPH into the basolateral side [106] greatly improving the relevance in estimating the first-pass metabolism in intestinal kinetics [79]. On the other hand, the presence of hydrolases associated with the microvillar membranes was verified in Caco-2 cells,

Table 1
Advantages and disadvantages of synthetic vs cellular models.

	ADV	DIS-ADV	REF
Synthetic models	• Low costs • Rapid to use • Easy to manipulate • Robust for testing lipophilic compounds • Capability to reproduce passive transport	• Poor prediction of paracellular route • No reproduction of intestinal epithelial morphology • No expression of transporting proteins • Absence of enzymes • Over-simplification of the system	[73,170]
Cellular models	• Capacity to mimic the *in vivo* anatomical, biochemical and structural features of the small intestine • Capability to reproduce both passive and active transport • Possibility to utilize human cells	• High variability related to cell differentiation and culture conditions • Longer times of preparation • Risks of contamination hampering the entire experiment • Higher costs	[7,21,82,170–172]

allowing to accurately mimic the brush-border enzyme activity of the small intestine [81].

Thereby, controversial outcomes and disagreements can be found in literature (Fig. SI-1). This could be due to the colonic cancerous origin of these cells, which show structural and functional features more similar to large intestinal cells than small bowel ones (e.g. stronger tight junctions, higher TEER values, altered enzymes and transporters expression) [104,107].

Furthermore, many works highlighted that Caco-2 cell-based systems are highly influenced by the experimental culture conditions, the cell line itself (i.e. the stage of cellular differentiation, whether the cells have reached confluency), and the passage number [108]. They are a heterogeneous population whose properties can differ between and within laboratories based on the different culture periods and the culture media used. Thus, it is fundamental to calibrate Caco-2-based experimental systems with reference compounds (chemicals with known absorbed fraction *in vivo*) and carefully control the environmental (temperature, pH) and barrier conditions (TEER) [78].

Currently, Caco-2 static monolayer cultures are a gold standard for *in vitro* assays of intestinal barrier properties following exposure to pharmaceutical and nutraceutical compounds [109].

It is commonly accepted that Caco-2 cells are the best intestinal epithelium model since they allow to exactly replicate the uptake via the transcellular pathway, which is the most common drug permeation route [14].

On the other hand, the active passage as well the metabolism of drugs in the gut is drastically underestimated [82,90]. Moreover, the absence of a mucus layer has a significant impact on this model fidelity, since it represents the first barrier that molecules meet, restricting their availability to the underlying cells [110].

3.2.2. TC-7 cell line

The TC-7 cell line is one of the Caco-2 subclones isolated to overcome the main limitations of the parental line, still maintaining its morphology (Fig. 2C) [111]. Among the several advantages of TC-7 cells, the faster cellular growth due to a shorter doubling time and the lower TEER values are certainly favorable features to more closely simulate the small intestinal epithelium [82,111]. Also, being a homogeneous population, less variability between and within laboratories was observed, ameliorating the robustness of the collected data [111]. Concerning the morphological features, the TC-7 monolayer revealed an increased height (15.4 ± 1.2 μm) respect Caco-2 cells (13.8 ± 2.4 μm) more similar to the native tissue (25 μm) [112].

Hence, TC-7 cells are very useful to analyze the transport biokinetics of chemicals. A good correlation between this subclone line and the Caco-2 cells was found for transcellularly absorbed drugs, indicating that the TC-7 model is an excellent alternative to Caco-2 monolayers [113,114]. Importantly, TC-7 cells also showed the ability to properly carry paracellular markers (e.g. mannitol, PEG-4000) via this passive route and a high content of apical transporters, being capable to even reproduce the carrier-mediated delivery, unlike Caco-2 cells [115].

Multiple brush-border enzymes resembling the human enterocytic metabolism were also observed, therefore eclipsing the original model [113]. The amount of UDP-glucuronosyltransferases (phase II metabolic enzyme), hydrolase sucrase-isomaltase and CYP3A isoenzymes (e.g. CYP3A4 and CYP3A5), which reached the culmination at late confluency, were noticed to be expressed very similarly to the human tract of jejunum [116] [108,117]. Furthermore, it was observed that also P-gp-mediated efflux occurred in a more *in vivo*-like manner than parental line [111,115].

Consequently, it is reasonable to consider TC-7 cells as a useful option for studying intestine first-pass metabolism and active absorption paths, differently from Caco-2 monolayers [118].

However, further rigorous investigations about their employment are desirable to better evaluate their large-scale screening potential, as little permeability data are available for TC-7 cells [115].

3.2.3. MDCK cell line

The Madin-Darby canine kidney (MDCK) is another epithelial cell line used for permeability measurements, which was isolated for the first time by Madin & Darby from a canine distal renal tissue [82]. Despite their different origin, MDCK cells are morphologically analogous to Caco-2 cells, imitating the *in vivo* intestinal barrier topology. In fact, these cells converge to form a polarized columnar monolayer displaying brush borders and tight intercellular junctions (173 ± 51 $\Omega \cdot cm^2$) (Fig. 2D) [118]. Two different strains of MDCK line were distinguished: (i) MDCK-I, which can generate a tight epithelium with high TEER values (above 1000 $\Omega \cdot cm^2$) and (ii) MDCK- II, which assemble a more permeable layer with lower TEER values (about 100 $\Omega \cdot cm^2$), thus being closer to the small intestine than Caco-2 cells [119]. In particular, these cells are less time-consuming (cells converge after 3-5 days of culture) than other epithelial cell models, consequently reducing the possibility of cell contaminations as well as costs [79]. For such benefits, MDCK cells were adopted to carry out rapid and bidirectional permeability screenings across renal and gastrointestinal epithelia during the early stages of drug discovery [119,120][118].

A clear relationship between this canine line and the human-derived Caco-2 one was demonstrated for passively absorbed drugs with a robust correlation (r^2=0.79) [121]. Furthermore, MDCK cells well correlate human absorption data (r^2=0.58), in line with Caco-2 cells (r^2=0.54) [121,122].

However, several shortcomings affect the experiments carried out by using MDCK cells. Firstly, as the monolayers do not tolerate great amounts of organic solvent, poorly soluble aqueous compounds are difficult to assess by using these systems. Moreover, the non-intestinal origin of these cells as well as their heterogeneity may hamper the reliability of the results depending on cells source, culture and transport conditions. The expression levels of transporters and the metabolic activity appeared also to be very different in the MDCK line respect the native scenario and the intestinal-derived cells [120].

To solve this lack, for example, the human MDR1 gene was transferred in the MDCK-II cells to obtain similar levels to the intestinal tract [7]. Interestingly, these transfected MDR1-MDCK cells overexpressed an isoform of P-gp as well as an enhanced polarized efflux of known substrates (e.g. digoxin) compared to other MDCK clones and Caco-2 cells [123].

Hence, MDR-1 transfected MDCK cells share many common epithelial cell aspects with the human intestinal mucosa and can be a helpful model for examining the carrier activity of drug candidates, such as for the P-gps. Nevertheless, their predictability and applicability remain very low compared to Caco-2 cells [124].

3.2.4. HT-29 cell line

HT-29 cells are a human colon adenocarcinoma cell that gained increasing attention due to the typical characteristics of mature intestinal cells [125]. HT-29 cells express features of both absorptive enterocytes and intestinal secretory cells producing a gelatinous mucus-like substance. For this reason, this cellular model is broadly employed to perform bioavailability studies or to investigate the intestinal immune response to bacterial infections that may affect the properties of the secreted mucus [126].

Notably, HT-29 cells phenotype strictly depends on the culture conditions, with particular attention to the presence or absence of glucose in the culture medium. These epithelial cells form undifferentiated and unpolarized multilayers without any typical features of intestinal cells under normal glucose supply conditions [127].

Conversely, HT-29 cells undergo intestinal differentiation patterns whether modifications of the culture medium or the addition of differentiation-inducers are used. For example, they can express several characteristics similar to the human enterocytes such as polarized monolayer structures, well-developed tight junctions, brush borders and microvilli when a glucose-free culture medium is used [128]. In addition, the presence of brush border-associated enzymes was observed in

enterocyte-like HT-29 cells [129]. Interestingly, most of them also express proper levels of villin as well as functional receptors for peptides and hormones existing *in vivo* [81,127,129]. Nevertheless, considering that not all hydrolases are present (e.g. lactase) and the enzymatic activity (e.g. hydrolases and sucrase-isomaltase) is lower than Caco-2 cells and the human small intestine, they cannot be properly considered as a reliable model of small bowel enterocytes [81,127]. On the other hand, they cannot be treated as colonic enterocytes since they express apical-localized hydrolases, which are normally absent in the colon [125]. The utility of this cell line is thus controversial also because several receptors naturally absent in the human epithelium (such as the neurotensin [130]) were detected in HT-29 cells, and others that are traditionally present (like that for the peptide YY or neuropeptide Y) have not been identified yet [126]. To surmount these constraints, other strategies to induce the enterocytic differentiation in HT-29 cells were carried out [131,132].

Strikingly, such efforts gave rise to other interesting lines: HT29-18-N_2, HT29-H and HT-29/16E. that produce a great number of mucins, accurately resembling the *in vivo* activity of goblet cells, thus being useful to examine the influence of the mucus layer on the intestinal absorption [81]. Especially, the HT29-H clone showed a variable thickness of the layer and a paracellular permeability higher and more similar to the native tissue with respect to Caco-2 monolayers [133].

In spite of this, HT-29 cells treated with methotrexate (MTX; HT29-MTX) currently remain the most used model to inspect how foods and xenobiotics alter the mucus and vice versa [134]. In particular, the levels of secreted mucins are influenced by the applied concentration of this drug. Low doses of MTX correspond to a heterogeneous set of both columnar absorptive and mucus-secreting cells, whereas mucus-secreting cells prevail by increasing the amount of MTX [126].

Moreover, HT29-MTX-D1 and HT29-MTX-E12 sub-populations were selected due to their ability to (i) develop thigh junctions, (ii) continuously secrete mucus and (iii) maintain stability for over 7 passages. Interestingly, it was noticed an inverse relationship between the permeability of lipophilic drugs (e.g. barbiturates and testosterone) and the thickness of the mucus layer. Especially, P_{app} values were lower in HT29-MTX-E12, which displayed a leakier but thicker mucus layer, than HT29-MTX-D1, which secreted mucus in smaller quantities [129,135]. Therefore, such cells revealed to effectively reproduce the mucus native properties.

Despite the common limitations due to the human colon carcinoma source, parental and sub-derived HT-29 cells proved to be a valuable model to screen endogenous and exogenous compounds [136,137]. The mucus-secreting phenotype received a huge interest in studies focused on food digestion and drugs bioavailability since this substance influences intestinal tissue homeostasis. Along this line, HT29-MTX cells appear to be the most suitable model due to their efficient mucus production (Fig. 2E), and for this reason, they are largely co-cultured with Caco-2 absorptive cells to create a more suitable *in vitro* intestinal-mimicking epithelium [138].

3.2.5. IEC cell line

The group of intestinal epithelial cells (IECs) is an intestinal rat cell line that can form pseudo-monolayers exhibiting microvilli, tight junctions and an amorphous substance similar to the basement membrane [82,139,140]. Even though their differentiation can be hardly induced *in vitro* since they derive from undifferentiated crypt epithelial cells, IEC cells treated with fetal rat or mouse gut mesenchyme become (i) absorptive, (ii) goblet, (iii) endocrine, and (iv) Paneth's granular intestinal cellular types [81]. The IEC line includes various cellular subgroups, such as the IEC-18, IEC-6 and IEC-14 line. Specifically, the IEC-14 cell line is very helpful in examining the regulation of crypt cellular proliferation and differentiation [139].

However, the IEC-18 subline is the most common intestinal barrier model traditionally utilized for investigating *in vitro* amino acids, glucose and other nutrients ingestion and digestion as well as cholesterol synthesis [118].

Importantly, the TEER of these cells (55 $\Omega{\cdot}cm^2$) was found to be comparable to the small intestine part of the ileum (88 $\Omega{\cdot}cm^2$), where paracellular permeation massively occurs [141,142]. As a consequence, it was confirmed that IEC cells better recapitulate the paracellular pathways in comparison to Caco-2 cells [143]. Indeed, paracellular hydrophilic markers such as mannitol, dextran and PEG-4000 demonstrated greater permeability coefficients across IEC-18 monolayers and molecular radius-dependence transport, unlike colonic cells [144]. Likewise, natural compounds such as the glycosides of genistein and daidzein (i.e. genistin and diadzin, respectively) crossed the intestinal IEC-18-based epithelium via paracellular delivery with a high absorption rate [145].

Hence, IEC-18 cells provide a size-selective barrier to *in vitro* discriminate and classify the hydrophilic molecules based on their molecular weights [141,146]. This is striking respect to Caco-2 cells that systematically underestimate paracellular uptake.

Conversely, uptake rates comparable to the *in vivo* scenario and Caco-2 cells were detected for lipophilic molecules transferred by the passive transcellular route, even though IEC-18 cells are less differentiated than Caco-2 cells [141,146]. Indeed, they possess less-developed tight junctional complexes and a slight polarization, and present a low expression of brush border enzymes (e.g. the sucrase-isomaltase or the intestinal isotype of alkaline phosphatase) [139,147]. This also results in an altered expression of carrier-mediated transport systems such as those of the MRP family. Besides, the quite absence of P-gps at the apical domain further correlate the low differentiated status of these rat cells with their crypt origin [141,144].

Given together, these outcomes suggest that this ileum-derived line is a useful *in vitro* cellular system for predicting the diffusion-mediated mechanisms of absorption, especially for hydrophilic compounds, which Caco-2 cells fail to anticipate. Notably, they precisely resemble the *in vivo* morphology and functionality of intestinal crypts rather than villus-localized enterocytes given the enormous lack of carriers and enzymes. As a result, these rat colon-derived cells may rapidly lose their markers of differentiations and their specific properties, thus being unstable and unpredictable [82,147].

3.2.6. HIEC cell line

The need to resort to a new type of intestinal cells to overcome the shortcomings originating from the cancerous nature of Caco-2 and HT-29 cell lines was addressed by employing human intestinal epithelial cells (HIEC). These cells represent a valuable option being capable to show some human crypt-like features [148]. Specifically, HIECs can form monolayers of polarized columnar cells with dense microvilli and poorly organized tight junctions, resulting in a morphology very similar to the *in vivo* context [149]. They exhibit low TEER values (98.9 $\Omega{\cdot}cm^2$) as wells as an aqueous pores size and distribution that determine the suitability of this line in replicating some of the crucial aspects and functioning of the human small intestine domain [150].

Accordingly, the existence of a sigmoidal relationship between F_a *in vivo* values and those measured *in vitro* for ten paracellularly delivered compounds was demonstrated, indicating a greater sensitivity and accuracy in anticipating the human paracellular uptake respect Caco-2 models. In particular, high permeability coefficients are systematically found for incompletely and poorly absorbable drugs with HIEC cells. Likewise, transcellularly transported drugs well permeate, highlighting the efficiency of this human-derived model in predicting the fractions absorbed in humans [151].

Furthermore, intestinal cell markers typical of undifferentiated crypt cells were found to be expressed in this line, such as low levels of intestinal enzymes (e.g. sucrase-isomaltase, alkaline phosphatase, CYP2C9, CYP2C19) [152,153]. Notably, other studies discovered low amounts of CYP3A4 (<7% of the human small intestine content) in HIECs and no considerable discrepancies between HIEC and Caco-2 monolayers for drug-metabolizing enzymes (e.g. CYP3A5) and

transporters (e.g. BCRP, MRP1, MRP2, and MRP3) [150].

Different stages of differentiation in HIEC cells can be achieved, replicating human crypt or enterocytes aspects. This may also vary based on the tissues from which HIEC cells derive. For example, Takenaka *et al.* investigated the characteristics and the possible applications of these epithelial cells differentiated from adult ISCs. Such cells, which possess a long-term proliferation and high-differentiating capabilities, generate absorptive enterocytes, but also goblet, enteroendocrine and Paneth cells under appropriate culture conditions [150]. Another alternative approach may be to obtain HIEC cells from the fetal human intestine at mid-gestation of a terminated pregnancy [154] or use fetuses derived by legal abortions, which have tissues with rapid metabolism and adult differentiated markers already expressed [148]. Nonetheless, despite their advantages, these cells are forbidden to use due to related ethical issues in many countries [148].

3.2.7. Co-culture models

The aforementioned cellular models remain far from reflecting the complex heterogeneity of the human intestinal organ [82]. For this reason, conventional Caco-2 cells are often *co-cultivated* with other cell types to generate more predictable models and thus perform effective drugs screening assays [118,155]. For example, the integration of Caco-2 cells expressing enterocytic markers with secretory cells, such as HT-29 sub-populations, may provide an environment that better mimics the *in vivo* conditions. Their contemporary presence can modulate the structure of the junctions to be more similar to those of the small intestine [120]. Indeed, Caco-2/HT-29 and Caco-2/HT-29-H mixed monolayers display a brush border membrane with tight junctions, and sparse irregular microvilli apically [156]. Moreover, a higher permeability closer to the F_a values was showed for passively ingested compounds respect the pure Caco-2-based system, providing an improved prediction of both transcellularly and paracellularly transferred drugs [157].

Similarly, the permeabilities of drugs absorbed via paracellular, transcellular and carrier-mediated pathways were evaluated by using three different initial seeding ratios of Caco-2 and HT29-MTX cells. Although no significant differences with Caco-2 pure layers were observed for the transcellular route, augmenting the fraction of HT29-MTX cells resulted in an increased uptake of compounds paracellularly delivered [155]. Interestingly, the P-gps expression in the co-cultures was detected at a lower level compared to the Caco-2 model, and an inverse relationship was noticed between the amount of P-gps and the fraction of the goblet-like cells [155,158].

Nevertheless, this cell mixture is not the only one employed so far. For instance, the Raji-B cell line derived from Burkitt's lymphoma recently gained attention, since these B-lymphocyte-like cells can induce Microfold cells (M cells)-like phenotype in Caco-2 cells [7,159]. Particularly, they are specialized epithelial cells playing a pivotal role in mucosal immunity by transporting antigens and pathogens to the underlying lymphoid tissue [120,160]. For these reasons, the Caco-2/Raji-B co-culture model revealed to be very useful in investigating the passage nanoparticles containing therapeutic peptides and vaccines [82,161,162], and explore both intestinal enterocytes and M cells absorptive capabilities [163]. Strikingly, the three cell lines maintain their function when cultured together, establishing an asset that can faithfully recreate the human intestinal barrier complexity [164]. Accordingly, these multiple cell cultures may be employed to obtain more physiologically relevant intestinal *in vitro* models to better evaluate and predict intestinal absorption mechanisms [165] (Table 2).

4. 3D cellular models

Recently, remarkable progress in developing 3D cell culture platforms have been achieved by mimicking fundamental physiological cues present in the *in vivo* native tissue. We will present an overview of the 3D static culture systems presently adopted in pharmacology research and industry discussing their advantages and limitations (Table 3) [179,180].

4.1. Villi-shaped scaffold for intestinal cell mono- and co-cultures of Caco-2 cells

Mimicking the complex geometry of small intestinal epithelium in traditional monolayers of mono- and co-cultures still remains a challenge nowadays. Therefore, several attempts oriented towards 3D scaffold-based models are spreading to more closely recapitulate the intestinal architecture (Fig. 3A) and thus perform more accurate pharmaceutical tests. Especially, scaffolds act as a support for cellular proliferation, differentiation and migration [181,182], playing a fundamental role in influencing cells behavior due to their chemical, mechanical and surface properties [183,184].

Among several materials and substrates, collagen-based hydrogels are the most widely employed for reproducing the shape of the villus-crypt axis. Caco-2 cells cultured onto these 3D structures (Fig. 3B) exhibit an increased absorptive surface area due to the villi-like scaffold conformation together with a proper expression of proteins that mediate the intercellular junctions, resulting in decreased TEER values and thus a more representative model of the human small intestine respect conventional monolayers [185,186]. Interestingly, it was reported that cell differentiation varied along the villus as it is observed *in vivo* [185].

In this context, since the paracellular route of absorption is well-known to be seriously underestimated in flat Caco-2 cells, several drug permeability studies investigating this pathway were performed to verify whether cells growth over the 3D villi-shaped scaffolds better resemble the intestinal barrier function. In particular, the paracellular transport of slowly absorbed drugs (FITC-dextran and atenolol) and rapidly absorbed drugs (fluorescein) showed higher permeability rates in comparison to 2D well-based cultures [181,186]. Specifically, atenolol permeability coefficients were 13 times higher in 3D models than planar Caco-2 cells, being approximately 60% of that measured in the perfused human intestine, thereby proving that these 3D structures can enhance the prediction of human intestine kinetics [172,181,185].

Moreover, the activity of metabolic enzymes and drug transporters in such 3D models was found to be more similar to the *in vivo* scenario [186]. For example, immunohistochemistry analysis revealed a quite low presence of P-gp proteins, while they were uniformly distributed and overexpressed in 2D cultures [185–187].

However, although collagen is suitable to support cellular growth and migration in a physically adequate 3D culture, this polymer can be subjected to batch variation, and its presence may limit the absorption rate of rapidly absorbed drugs. To resolve these concerns, investigations about alternative materials were proposed to recapitulate villi and crypts architectures without interfering with the permeation process [185,188].

Patient *et al.* fabricated polyethylene terephthalate nanofibrous scaffolds to mimic the intestinal epithelium topography. As for collagen, TEER values lower than those observed in 2D Caco-2-seeded Transwell were reported, probably due to the increased porosity of nanofibers over monolayers. Furthermore, the bidirectional absorption of FITC-dextran, lucifer yellow, rhodamine 123, propranolol, and atenolol displayed higher permeability through Caco-2 cells cultured on 3D scaffolds compared to 2D typical platforms [187].

Hence, it was widely accepted that the 3D corrugated structure fosters the establishment of a physiologically operating barrier very close to the native gut epithelium that increases the potential of the test model in predicting the intake of pharmaceutics both via passive and active pathways, regardless of the scaffold material used [188,189].

Interestingly, the ability of these models to successfully support co-cultures of Caco-2 and HT29-MTX cells with prominent stromal cells (i.e. fibroblasts and immunocompetent macrophages) was also explored. This setting-up demonstrated a 3D larger absorptive surface area, decreased TEER values and a viscous mucus layer formation. This more

Table 2
Characteristics of cellular models.

Model	CACO-2	TC-7	MDCK	HT-29	CO-CULTURE	HIEC	IEC
CELL SOURCE	Human colon adenocarcinoma cell line	Caco-2 subclone	Canine distal renal tissue-derived line	Human colon adenocarcinoma cell line	Human colon adenocarcinoma cell lines	Small intestinal crypt-derived human cell line	Small intestinal crypt-derived rat cell line
MORPHOLOGY	Polarized monolayer with strong tight junctions, apical brush borders and microvilli	Polarized monolayer with tight junctions, apical brush borders and microvilli	Polarized monolayer with tight junctions, apical brush borders and microvilli	Polarized monolayer with tight junctions, apical brush borders with sparse and short microvilli or a mucus layer (based on culture medium content)	Polarized monolayer with tight junctions, apical brush borders, microvilli and mucus layer	Polarized monolayer with poorly organized tight junctions, apical brush borders and dense microvilli	Polarized monolayer with poorly developed junctions, brush border, apical microvilli and an amorphous substance similar to the basement membrane
TEER [$\Omega \cdot cm^2$]	250-2500	150-750	173 (parental line); ~1000 (MDCK-I subgroup); ~100 (MDCK-II subgroup)	15 (undifferentiated HT29 cell line); 280 (HT29-MTX-D1 subclone); 170 (HT29-MTX-E12 subclone)	250-400 (50% CACO-2/ 50% HT29-MTX); 60 (CACO-2/HT-29/Raji-B)	98.9	55 (IEC-18); 100 (IEC)
ENZYMES & TRANSPORTERS	• High expression of P-gp (MDR1), MRP2, MRP4 and BCRP, OATP1B1, OATP1B3, OATP2B1, PepT1, OCT1, OCT2 and OCT3 • Absence of CYP isoenzymes (possibility to induce CYP3A4 expression with 1-α-2,5-dihydroxyvitamin D3 or NADPH) • Proper expression of brush border enzymes	• High expression of P-gps • Expression of UDP-glucuronosyltransferases, hydrolase sucrase isomaltase • Large quantity of CYP3A enzymes (CYP3A4 and CYP3A5)	• Minimal expression of P-gp (solved with MDR1-MDCK cell line) • Expression of MDR1, MRP1, MRP2 and MRP5	• Low expression of brush border enzymes (e.g. forms of hydrolases) • Absence of lactase	• Proper expression of P-gps	• Expression of BCRP, MRP1, MRP2, and MRP3 • Expression of CYP3A5 enzyme • Low amounts of CYP3A, CYP2C9, CYP2C19, sucrase-isomaltase and alkaline phosphatase	• Altered expression of carrier-mediated transport systems (e.g. MRP family) • Poor expression of P-gps • Low expression of brush border enzymes (e.g. sucrase-isomaltase, alkaline phosphatase)
ADV	• High accuracy in replicating the enterocytic phenotype • Long- term cell viability • Great reproduction of the transcellular pathway • Good reproduction of the active pathways	• Fast cellular growth • Great homogeneity ensuring consistent results • Good reproduction of the passive pathways • Good correlation with Caco-2 for passively transported drugs • Possibility to study intestinal metabolism	• Short culture time (3-5 days) • Low costs • Possibility to obtain several sub-clones • Good reproduction of the passive pathways	• No expression of P-gps • Possibility to obtain several sub-clones by treating the culture medium with specific substances • Possibility to obtain goblet-like cells Possibility to study the mucus layer	• Reproduction of both absorptive and secretory epithelial cells • Mucus production • High permeability for passively absorbed drugs • Possibility of modifying the permeability barrier of the cell monolayer (flexibility) • Good reproducibility	• Pore sizes and distribution similar to the human small intestine • Long-term proliferation capability • Ability to differentiate into several cell types • Great accuracy in predicting the human paracellular uptake	• Non-cancerous origin • Intestinal origin • Good reproduction of the paracellular path • Useful model for studying cholesterol synthesis and the role of growth factors in epithelial layers
DIS-ADV	• Colon cancer origin • Long differentiation time (~3 weeks) • Lack of physiological factors (mucus, bile salts, cholesterol) • Inter- and intra-laboratories variability • Underestimation of paracellular transport	• Absence of large-scale data	• Non-human origin • Non-intestinal origin • High variability • Very low toleration of great amounts of organic solvent	• Cancerous origin • Non-small intestinal origin • Altered glucose metabolism • The differentiation process occurs only under specific culture conditions and require a long time • Absence of the major metabolizing proteins	• Cancerous origin Difficulties in manipulating and maintaining different cell lines contemporarily	• Crypt origin • Interindividual variations • Ethical issues prohibiting their use in several states	• Non-human origin • Crypt origin • Long differentiation time (~3 weeks) • Instability (rapid loss of differentiation markers) • No transporting proteins
REF	[7,85,177,178]	[113,177]	[7,124,175–177]	[120,135,174]	[120,138,155,173]	[150]	[7,141]

Table 3
Characteristics of 3D models.

3D Static Model	Villi-shaped scaffold for intestinal mono- and co-cultures	Organoids	Epi-Intestinal
MORPHOLOGY	Structure with an increased absorptive surface area due to the villi formation; tight junctions and a mucus layer in co-cultures are present	Highly-folded differentiated epithelium with a central hollow region composed of differentiated cells (goblet, enteroendocrine cells, enterocytes) extruded into the lumen creating villi domains and apex regions (ISCs and Paneth cells)	Wall-to-wall polarized microtissue; highly differentiated epithelium including enterocytes, Paneth cells, M cells and ISCs with villi, microvilli, tight junctions, brush borders and a mucus layer
TEER [$\Omega \cdot cm^2$]	40-80 (3D hydrogel scaffold); 220 (3D nanofibers);	Not quantifiable	130-192
ENZYMES& TRANSPORTERS	• Lower presence of P-gps and higher expression of transporters (e.g BCRP, MRP) respect to 2D monocultures	• Expression of GLUT2, GLUT5 and PEPT1 transporters • High expression of CYP3A4, CYP3A5, CYP1B1, CYP2B6 and CYP2J2 enzymes	• Expression of P-gps and MRP-1, MRP-2, BCRP • Presence of most relevant enzymes (CYP3A4, CYP3A5, CYP2B6, CYP2C9, UGT)
ADV	• Easy access to apical and basolateral sides • 3D large absorptive surface area • High production of mucus • Accurate reproduction of the intestinal villus-crypt morphology • Use of biodegradable and bioactive material	• Self-organization • Self-renewal • Possibility to expand indefinitely • Long-term culture (1 year or more) • Absence of cancerous cells • Reproduction of the intestinal cellular heterogeneity • Reproduction of the villus-crypt morphology	• Easy access to apical and basolateral sides • Long-term culture (up to 6 weeks) • High reproducibility • Low lot-to-lot tissue variability Relatively inexpensive
DISADV	• Scaffolds may limit the absorption rate • Hydrogels are subjected to batch variations • Relatively complex fabrication process	• Closed and hardly manipulable lumen • Absence of a vascularization • High costs • High variability (source, size, shape) • Complex experimental techniques • Need for primary cells	• Single unknown donor origin Polymorphic metabolizing enzymes not representative of a population
REF	[85,113,177,178,185]	[201,204,205,207,221,227,228]	[85,219–221,226]

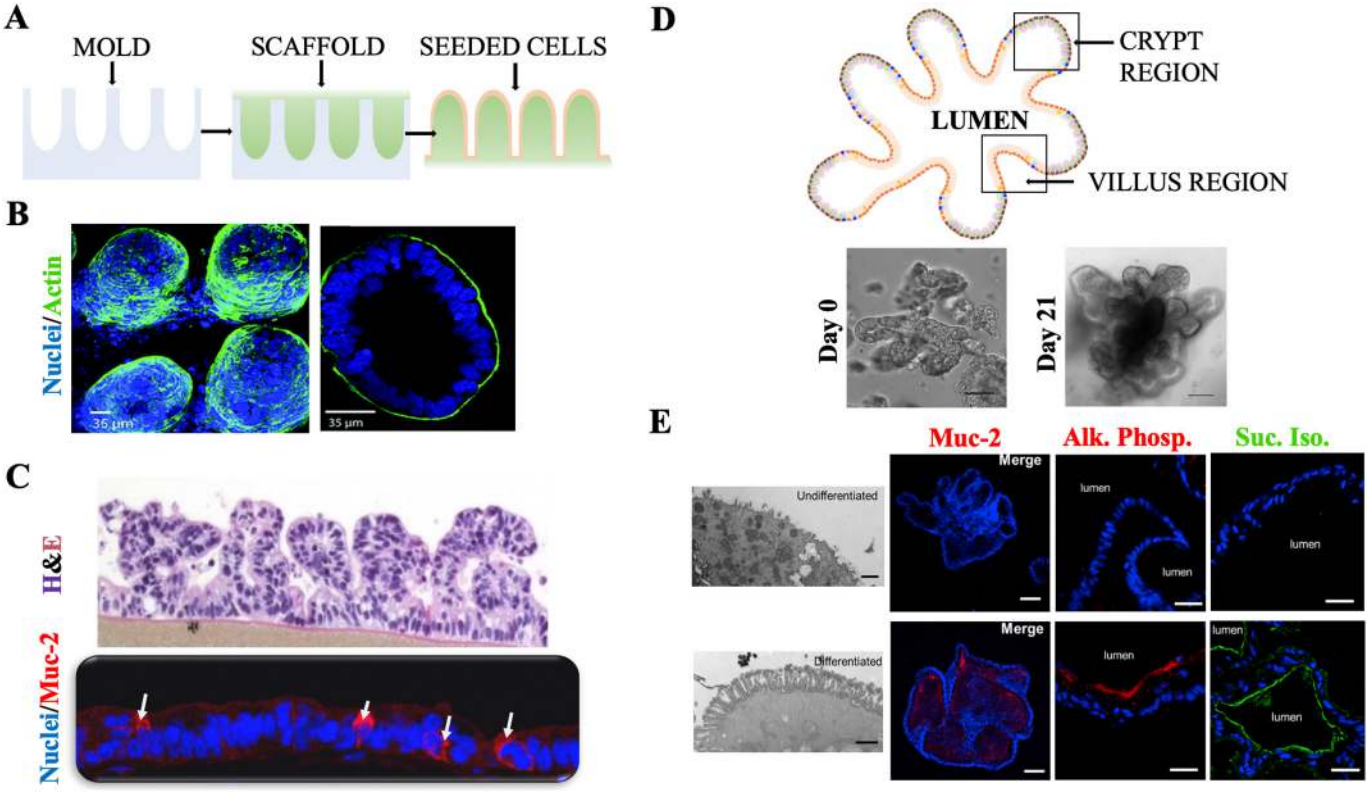

Fig. 3. Intestinal 3D static culture systems resemble crucial features of differentiated intestinal tissues. (A) Schematic representation of villi-shaped scaffolds fabrication. (B) Confocal image of Caco-2 cells cultured on a collagen-based scaffold (left); hydrogel horizontal slice showing cells properly polarized and uniformly seeded on the scaffold (right); republished with permission of Royal Society of Chemistry, Copyright (2010), from [223]. (C) Illustration of intestinal organoid topography with villus-protrusions towards the lumen and crypt regions (top); such architecture can be obtained *in vitro* by culturing human intestinal tissue-derived crypts (bottom, left) after 21 days (bottom, right); scale bar: 100 μm [224]. (D) Differences in Muc-2 production, Alkaline Phosphatase, and Sucrase Isomaltase expression in undifferentiated (top) and differentiated (bottom) intestinal organoids. Scale bars = 1 μm for TEM images and 50 μm for microscope images; reprinted and adapted from [225], Copyright (2016), with permission from Elsevier. (E) Typical histology (top) and immunohistochemistry image (bottom) of the Epi-Intestinal^{TM} (SMI-100) tissue illustrating an *in vivo*-like 3D structure and the simultaneous presence of both mucus-secreting and absorptive-like cells after 14 days of culture, respectively [219].

physiological morphology culminated in a decreased permeability for moderately or highly absorbed drugs (propranolol, verapamil, antipyrine and carbamazepine), and an increased uptake for low absorbable compounds (ranitidine and furosemide) with a high correlation with human data (r^2=0.84) [190].

Taken together, these outcomes denote 3D scaffold-based intestinal mucosa models as powerful and versatile systems to resemble the most important native features and functions, with a strong potential in drug screening experiments under static conditions [191]. However, additional studies should be conducted to select other materials with more suitable biochemical and mechanical properties to generate an *in vivo*-like intestinal epithelium. It would be interesting also to include other intestinal lines and culture organoids-derived stem cells, as well as to investigate whether biofabricated 3D structures or decellularized small intestinal submucosal scaffolds could control intestinal cells differentiation, proliferation and transport activity [189,192].

Finally, future developments should include fluid flows and mechanical stimulations as it happens in the living intestine [193].

4.2. Organoids

Organoids are 3D organ-buds created *in vitro* that have a realistic microanatomy due to their *in vivo*-like self-organizing and self-renewing capabilities [183]. They brought notable ameliorations in tissue engineering by exhibiting levels of cellular complexity similarly to native organs, bridging the gap between 2D monolayers and animal testing [163,194]. To date, several 3D organotypic cultures replicating various organs involved in the GIT tract, such as the pancreas [195], stomach [196] and intestine [197], have been developed. Especially, small intestinal organoids, also called mini-guts or enteroids, are advanced technologies that are currently galvanizing the *in vitro* modelling to investigate physio-pathological mechanisms. Mini-guts can replicate the *in vivo* tissue morphology and physiology by retaining key intestinal physiological conditions and functions (e.g. crypts and protruding villi formation, cytochrome P-450 metabolizing activity, mucus secretion) for prolonged periods (Fig. 3C, D) [163,198,199]. They have a central hollow region made up of differentiated cells (e.g. goblet cells, enteroendocrine cells, enterocytes), which are extruded into the lumen forming villi- and microvilli-like domains, and an apex region where ISCs and Paneth cells reside, giving rise to crypt bases [194,200].

Enteroids are usually derived from primary tissues (either from a single ISC-expressing Lgr5 marker or isolated intestinal crypts [201–203]) or also from pluripotent stem cells (PSCs), both in the form of induced (iPSCs) and embryonic (ESCs) cells [204], which can differentiate into all cell types of the adult intestine thus creating a multilineage culture system [205]. Notably, in the personalized medicine scenario, intestinal patient-derived stem cells-induced organoids are very useful for establishing a patient biobank and a specific platform where to evaluate therapeutic strategies preventing any immune response [206].

However, although these 3D cellular structures faithfully resemble the human small intestine complexity, their morphology and architecture may represent a significant obstacle for studying the intestinal barrier absorptive function due to their geometry [207]. The closed lumen precludes direct access to the apical surface hampering PK assessments of endogenous and foreign substances [207,208]. It's particularly difficult to manipulate entrapped cells and conduct quantitative analyses of the transcellular and paracellular diffusion and the presence of apical carriers without altering the organoid architecture [209]. For these reasons, alternative methods to access the luminal side, such as the microinjection technique, have been conceived, particularly for examining transport phenomena of foods, toxins and drugs across the intestinal epithelium [207]. For example, the translocation of monosaccharide compounds (e.g. glucose and fructose) and peptides (e.g. Gly-Sar) was investigated by adopting this strategy and conjugating a fluorescent tracer to the substance of interest [204,210,211]. Nonetheless, the excessive costs of fluorophores and the limited reproducibility of the microinjection, which usually requires the use of micromanipulators, hinder its applicability to organoids for studying substances permeation [62,204]. Moreover, this strategy gained restricted success as it also provokes irreparable damages to the tissue [212].

Another solution was identified in mechanically disrupting the organoid 3D structure and then replate recovered cells in traditional 2D plates to obtain well-differentiated intestinal multilineage cells in an easy-to-use conformation, allowing the direct manipulation of the apical and basolateral side of the tissue [213]. Recently, this method was adopted by Yoshida *et al.* for evaluating the entry and metabolism of pharmaceutical compounds [214]. In particular, once an organotypic culture was established from human iPSCs, it was subsequently mechanically dissociated. Then, researchers purified the culture from mesenchymal cells, which could influence the screening activity of the model, cultured the gathered iPSC-derived IECs as a monolayer [197,214]. The potential of the established iPSC-IECs barrier was confirmed by the proper metabolization of terfenadine and midazolam, which are representative substrates of CYP3A and CYP2J2 as well as CYP3A enzymes, respectively, and cannot be reproduced with conventional 2D models. This technique revealed to be also valid to assess the uptake of other molecules, proving the reliability of the organoid-derived epithelial monolayers for investigating xenobiotics absorption [104,214]. However, the dissociation process may disrupt the stem-cell compartment and thus the continuous propagation ability of the organoid. In addition, the authors suggested that a refinement of the presented procedure is still necessary to ensure its reproducibility and replicability to perform high-throughput assays [214].

Hence, mini-guts are not the easiest-to-handle tools for investigating transport processes through an intestinal barrier model. Moreover, the absence of vasculature, which is fundamental for nutrient and waste transport, as well as fluid and mechanical stimuli (e.g. luminal flow or peristalsis motions) remarkably impact the reliability of the model [194,197]. To close this gap, a possible strategy may consist of integrating these enteroids with dynamic culture systems, such as commercial bioreactors or custom-made fluidic devices, where physical, biological and chemical parameters could accurately be imposed [215,216].

Nevertheless, the elevated costs for the enteroid formation and maintenance must be also considered in a pre-clinical view. In this scenario, it is also well-known that outcomes derived from drug toxicity and efficacy assays, performed by using intestinal organoids, often lack reproducibility because of the intrinsic variability of the organoids source, size and shape [7,215]. All these aspects may lead to significant difficulties in gaining robust statistics of the obtained results, such as PK profiles of new drug candidates, impairing the translational potential of mini-guts as preclinical screening platforms [62]. Therefore, further efforts are certainly necessary to consider these promising organotypic systems in an increasingly present perspective of reducing as much as possible animal testing in drug discovery and development [217].

4.3. Human Reconstructed small intestinal tissues

Epi-Intestinal™ is an innovative 3D human reconstructed gut tissue model developed by MatTek Corporation which closely recapitulates several cues of the native small intestinal barrier. Indeed, it was demonstrated to be a functional biologically-relevant tool in a wide range of applications, such as drug absorption and metabolism as well as GIT toxicity and inflammatory studies [85,218]. This recent technology is emerging in the GIT *in vitro* research field thanks to its proven advantages over the other cell-based systems currently employed [219,220]. In fact, Epi-Intestinal, like enteroids, comprises cellular heterogeneity deriving from primary intestinal cells, which makes the reliability of data more solid compared to 2D immortalized cell-based

models (Fig. 3E). In particular, it includes enterocytes, Paneth cell, M cells, and intestinal stem cells into a polarized epithelium, precisely replicating the architectural and phenotypic features of the small intestine.

Moreover, this organotypic microtissue possesses an open physiological luminal surface which is extremely functional in investigating drug and nutrients processing, differently from the closed geometry of organoid-based models. Importantly, this topography allows easy access to the lumen compartment which is highly profitable for bidirectional permeation studies both from the lumen to the bloodstream and vice versa [221].

To demonstrate its analogy to the human small intestine, other primary features of the Epi-intestinal were examined. For example, Ayehunie and colleagues demonstrated the formation of villi, microvilli, tight junctions and brush borders similar to the *in vivo* scenario by performing immunohistochemistry analyses [85]. The genetical expression of metabolizing enzymes and both uptake and efflux transporters were carefully determined with selective substrates and inhibitors. Results revealed the presence of MRP-1 and MRP-2, BCRP, and the main drug-metabolizing enzymes (CYP3A4, CYP3A5, CYP2B6, CYP2C19, CYP2C9 and UDP-glucuronosyltransferases (UGT) with very little biological differences respect the human situation [85,220].

These outcomes supported the enhanced predictability potential of the Epi-intestinal system compared to Caco-2 cell cultures, where CYP3A4 and several drug transporters are almost absent or low expressed [220]. The presence of these relevant transporters and enzymes made Epi-intestinal a suitable model also for bioavailability studies of many drugs (including talinolol, ranitidine and warfarin) with different physicochemical properties. In this context, the Epi-intestinal system was found to be very useful to rank order compounds as low (<50%), moderate (50-84%) and high (≥85%) absorbed drugs [221]. Along this line, a good correlation between the first-pass GIT availability ($F_a \times F_g$) calculated *in vitro* with that of the human PK values was noticed for a panel of twelve drugs (e.g. atenolol and midazolam) [220].

The inability of cancerous cells-based systems in predicting human absorption was emphasized in comparison to Epi-intestinal technology. Data measured by using the organotypic intestinal tissue displayed a higher correlation with the human fractions (r^2=0.91) respect Caco-2 cells (r^2=0.71) [85,221]. Furthermore, high reproducibility and low lot-to-lot tissue variability for permeability assays were demonstrated, as opposed to traditional monolayer cultures [85,222].

Therefore, Epi-intestinal microtissue represents an optimal 3D model to investigate drug absorption, by mimicking morphological and functional aspect of the small intestine. Even though it provides multiple advantages over the other available *in vitro* systems, some limitations hamper its use in pharmaceutical research. For example, like other 3D tissue models, Epi-intestinal is not provided with an inner vascularization and is usually employed under static incubation conditions, which do not resemble the physiological fluidic stimuli of the *in vivo* microenvironment [219]. Moreover, the lack of dynamic recycling of the drugs may underestimate their bioavailability, especially for drugs that are extensively metabolically cleared [220]. To our knowledge, only Marrella *et al.* proposed a novel approach to resolve this issue by integrating the 3D Epi-intestinal tissue into a commercialized bioreactor to better mimic the physiological fluid-dynamic microenvironment of the gut [219]. In addition, such microtissue derives from a single donor and thus it cannot be representative for a population with polymorphic metabolizing enzymes [220].

Hence, even if Epi-Intestinal is a leading *in vitro* technology widely accepted by formulation scientists and toxicologists as an excellent animal alternative platform for absorption and toxicity assays of pharmaceutical and nutraceutical products, additional attempts examining a larger set of compounds need to be carried out.

5. 3D fluid-dynamic *in vitro* models of the human intestinal barrier

3D fluid-dynamic *in vitro* models are promising tools having the potential to closely reproduce the complex physiology of the human intestine, overcoming the limits that affect static cultures. In the gastroenterology research field, they provide critical physicochemical aspects that are crucial in emulating the intestinal activity, faithfully recreating an *in vivo*-like dynamic microenvironment [229]. In fact, fluid flows and mechanical stimuli were demonstrated to tremendously alter the gene expression profiles (23.000 genes targeted) compared to static systems [230]. Herein, we provide an overview of the existing intestine-on-chips and, more in general, of the fluidic platforms aimed at recapitulating the human intestine physiology with an organ-scale level for absorption and metabolism investigations. OOCs of the human intestine usually consist of 2 channels, emulating the intestinal lumen and the vasculature, separated by an ECM-mimicking gel-coated porous membrane over which intestinal epithelial cells can be cultured (as represented in Fig. 4A-B-C). Frequently, Polydimethylsiloxane (PDMS) is used to realize these chips since it is gas-permeable and allows fast prototyping and high-resolution imaging [62]. In these systems, dynamic culture conditions are established by imposing tailored fluid flows; in this way, the influx and efflux of substances present in the chyme and the bloodstream can be investigated across the intestinal-simulating barrier [62,207].

Interestingly, although immortalized cell lines (e.g. Caco-2 cells) displayed several limitations under static culture conditions, they can develop a compact intestinal epithelial layer with 3D villi-like structures and basal crypts when properly stimulated by a fluid-dynamic active environment [179,231–234]. Moreover, Caco-2 cells complete their differentiation already after 5-7 days under fluid flow, unlike planar monolayers that exhibit a complete mature phenotype after 21 days (Fig. 4B) [56,179,233,235,236].

When the fluidic stimuli are also combined with mechanical cyclic strains to perform peristalsis-like deformations, intestinal cells undergo a spontaneous differentiation reprogramming themselves into 4 different small intestinal cell types (enterocytes, enteroendocrine, Paneth and Goblet cells), presenting a columnar *in vivo*-like cell morphology [179].

In this context, several studies aimed at investigating xenobiotics and food-related entities transport across the intestinal epithelium were performed and compared with traditional Transwell- or Ussing chamber-based cultures. For example, Kulthong *et al.* carried out biokinetic studies of high (antipyrine, ketoprofen and digoxin) and low (amoxicillin) permeability compounds in the two opposite culture conditions. Results revealed that a lower drug uptake occurred in the gut-on-a-chip with respect to the static condition with permeability values in line with the BCS. Similarly, the absorption rates of several compounds (curcumin, mannitol, dextran, caffein and atenolol) in Caco-2 cells-seeded microfluidic devices were reported to be comparable with human data [56,231,234]. Moreover, under dynamic stimulation, Caco-2 cells revealed to be efficient to *in vitro* inspect and reproduce PK profiles of orally-delivered chemotherapeutic agents, which often fail to pass the intestinal barrier because of their low solubility and permeability. New insights were provided for lipophilic chemotherapeutic prodrugs such as SN-38 (7-ethyl-10-hydroxycamptothecin) and approved antineoplastic drugs like 5-FU [56,237]. In particular, for this latter, a concentration-time curve with an *in-vivo* like pattern of absorption and excretion was generated by applying a peristaltic flow on the top channel, and its therapeutic effect on target lung cancer cells was verified within the same chip.

Therefore, it is clear that fluid flows significantly regulate the transport of drugs, for example limiting their diffusion due to the recirculation of the luminal content. Dynamic conditions trigger a 3D intestinal *in vivo*-like morphogenesis, increasing the available absorptive surface and, consequently, the absorptive efficiency of the tissue [238].

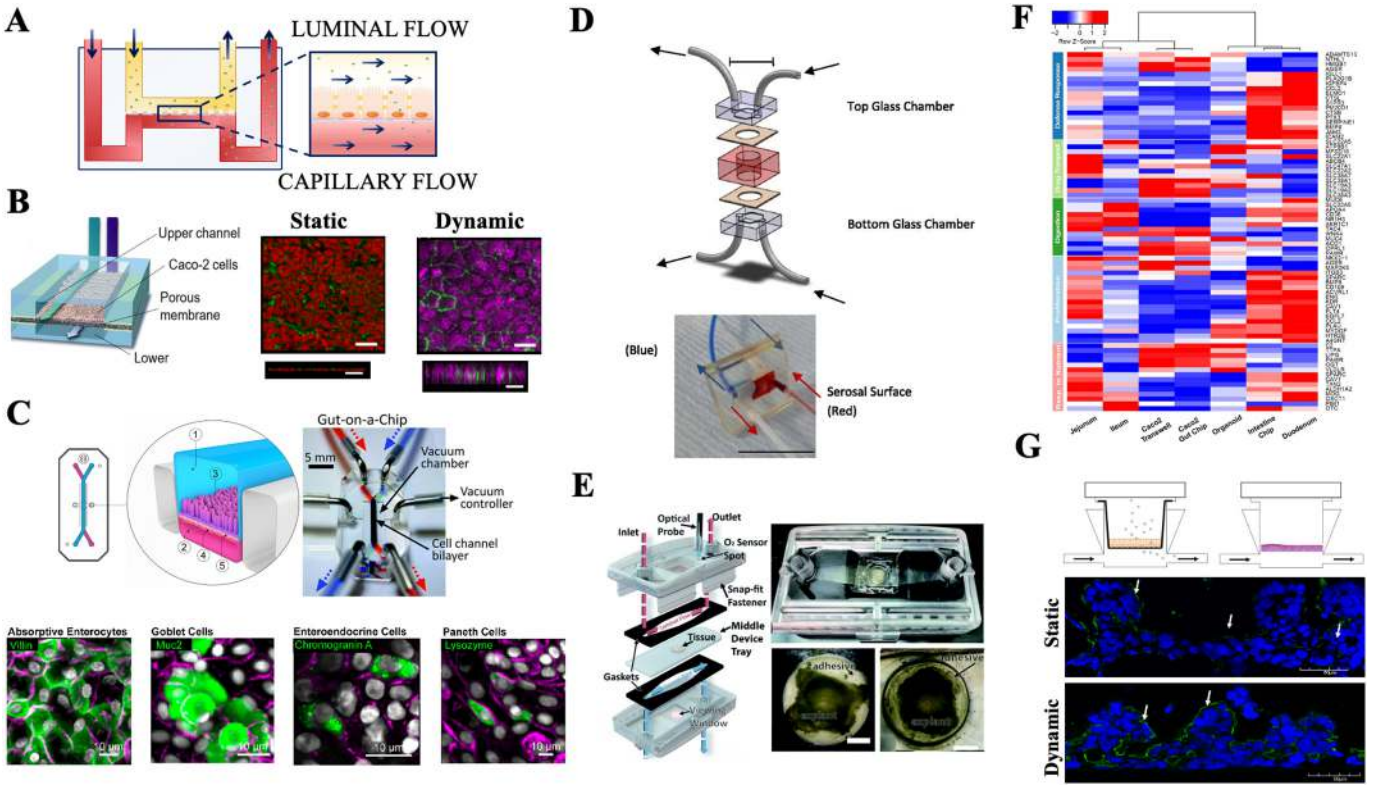

Fig. 4. Intestinal OOCs provide a physiologically relevant environment: the human intestinal epithelium can be accurately recapitulated *in vitro*. (A) Typical design of an intestine-on-a-chip composed of two channels mimicking the lumen and the blood circulation, separated by a membrane over which cells are cultured. (B) 3D rendering of Caco-2 cells seeded microfluidic chip (left); immunostaining of Caco-2 cells cultured in a Transwell system (middle, static condition) at day 21 and the microfluidic device (right, dynamic condition) on day 3. Fluid flow stimulates the formation of an undulating 3D morphology with brush borders. The height of the monolayer reached 40 μm – 50 μm on day 3 when fluidically stimulated (bottom); scale bar = 50 μm [231]. (C) Schematic representation (top left, [216]) and image (top right, republished and adapted with permission of Royal Society of Chemistry, Copyright (2012), from [236]) of the "Intestine Chip" by Emulate Inc. consisting of two channels separated by a gut-mimicking epithelium and surrounded by two lateral vacuum chambers which apply a cyclic strain to the cells; confocal fluorescent micrographs confirm that fluidic and mechanical stimuli induce organoids-derived cells differentiation into several intestinal cell types after 8 days of culture within the chip (bottom) [216]. (D) Exploded design of the dual flow device able to hold and perfuse a full-thickness intestinal tissue section (top); Red and blue dyes demonstrating the dual independent fluidic circuits (bottom); reprinted from [243], with the permission of AIP Publishing. (E) Schematic illustration of a microfluidic chip capable to maintain an intestinal tissue explant ex vivo in a physiologically relevant environment (left); Image of a mouse colon explant inside the device (right, top) at 0 h and 72 h through a viewing window (right, bottom). Scale bars: 2 mm; republished with permission of Royal Society of Chemistry, Copyright (2020), from [244]. (F) A heatmap of gene expression profiles comparing *in vivo* tissues (jejunum, ileum, duodenum) and *in vitro* reconstructed epithelial models (Caco-2-based Transwell, Caco-2-based chip, 3D organoid, organoid-based intestine chip). Combining organoid-derived epithelial cells with fluidic and mechanical stimulations allows to better recreate intestinal organ-level physiology [247]. (G) Schematic representation of the MIVO® fluidic system hosting either a Caco-2 monolayer and the EpiIntestinal™ (SMI-100) tissue and resembling the capillary flow below the gut model (top); immunohistochemistry analysis demonstrating that EpiIntestinal™ (SMI-100) tissue develops an *in vivo*-like morphology (presence of villi and microvilli) when properly stimulated by a biologically relevant fluid flow [219].

In fact, it was recently demonstrated that fluid flow-associated forces such as shear stress play a pivotal role in epithelial cells growth and microvilli formation [239,240].

Notably, Caco-2 cells secrete mucus, unlike standard static cultures in microfluidic chips [179]. Furthermore, it was shown that luminal and basal flows are responsible for the proper expression of transporters on the apical and basolateral side of the barrier [231]. Likewise, biomimetic chips promote the establishment of the main intestinal metabolic pathways, differently from Transwell systems, where cells lack the expression of the majority of enzymes [232]. For example, 3D collagen-based scaffolds seeded with Caco-2 cells were integrated within the chips to couple a 3D intestinal topography with fluid flow patterns [193,241]. However, hydrogels are challenging to inject in microdevices at low concentrations, and, at the same time, increased volumes subsequently alter the stiffness of the mimetic matrix. For this reason, this technique is currently rarely used [62].

In addition, it is necessary to keep in mind that Caco-2 cells exhibit a specific mutated genetic profile also when fluidically stimulated [242].

Therefore, another valuable approach was identified in incorporating mammalian full-thickness explants in dual-flow platforms [243–245] (Fig. 4D-E). Despite the promising results obtained in faithfully mimicking all aspects of the intestinal pathophysiology, their employment still remains extremely restricted due to the remarkable difficulties in their usability and maintenance in culture, hampering the possibility to perform pharmacology assays [207,243].

To date, advancements in the GIT research and the hopeful outcomes arising from the microfluidic devices determined the circumstances to recreate intestinal organ-level physiology by combining organoids and fluidic devices. In this scenario, some researchers were able to culture primary stem cells (derived from fragmented enteroids) within these platforms, overcoming the challenges shown by human organoids in performing screening assays due to the enclosed geometry, as discussed before [246]. In this way, a well-formed intestinal epithelium with tight junctions, brush borders and elongated villus-like protrusions, as well as a complete intestinal differentiation can be developed in response to fluidic and mechanical stimulations within 8-12 days [246–248]. Fluorescence microscopy confirmed the successful maturation of all major intestinal epithelial cell types, such as proliferative cells in the basal

Table 4
Characteristics of fluid-dynamic models.

Model	Cell source	Features	Adv	Dis-adv	Ref
Gut-on-a chip	Cell lines (mono- and co-cultures)	PDMS-based chip with 2 channels reproducing the intestinal lumen and the vasculature, separated by an ECM-mimicking gel-coated porous membrane	• Mucus layer • Formation of 3D villi-like structures • Reproduction of physiological levels of fluid flows and shear forces • Spontaneous and rapid differentiation due to the dynamic stimuli (5-7days)	• Possible non-specific binding of hydrophobic molecules onto PDMS surface • Altered gene expression profiles	[231–235]
3D scaffolds on chip	Cell lines (mono- and co-cultures)	Microfluidic device (PDMS sheets with fluidic channels) integrated with 3D hydrogel-based villi-like scaffold	• Combination of a 3D villi geometry with fluid flows positively impacting cell differentiation	• Possible non-specific binding of hydrophobic molecules onto PDMS surface • Scaffolds may limit the absorption rate • Hydrogels are subjected to batch variations • High complex fabrication process	[193,241]
Microfluidic organotypic device	Full-thickness intestinal tissue section and tissue explant	Dual flow perfusing full-thickness intestinal tissue sections or tissue explants *ex vivo* in a physiologically relevant environment	• Biomimetic and organotypic intestinal culture models • Simultaneous presence of muscular, neural, immune and epithelial components • Double fluid flows mimicking the intestinal lumen and the vasculature	• High costs • Short-term assays (72 h) • Poor scalability and repeatability	[243,244]
"Intestine Chip" by Emulate Inc.	Cell lines (mono- and co-cultures), organoid-derived cells	PDMS-based chip with 2 channels reproducing the intestinal lumen and the vasculature, separated by an ECM-mimicking gel-coated porous membrane and supported with two lateral vacuum chambers which apply a cyclic strain to the cells	• Combination of organoid-derived intestinal cells of three independent donors with microfluidic chips technology • Well-formed *in vivo*-like intestinal epithelium • Simultaneous fluidic and mechanical stimulations • Proper gene expression profiles	• Longer intestinal differentiation (8-12 days) • Complex experimental techniques • High costs	[216,236,247]
"MIVO" by React4life	Cell lines (mono- and co-cultures), organoid-derived cells, biopsies, 3D commercial reconstructed tissues	Optical-transparent *in vitro* multi-chamber platform easily accommodating 2D cell monolayer or 3D human tissues and biopsies with clinically relevant sizes	• Physiological mass transport and remotion of waste due to the dynamic fluid flows • Clinically relevant sizes of tissue model • Possibility to study the passage of molecules across the healthy or pathological small intestinal barrier	• Use of a greater volume than microfluidic chips • Low throughput	[22,219]

regions, mucin-producing cells, enterocytes covered with densely-packed microvilli, and enteroendocrine and Paneth cells [216,247,248] (Fig. 4C).

Strikingly, a human intestinal endothelial-parenchymal tissue-tissue interface was also be successfully recapitulated by seeding human intestinal microvascular endothelial cells (HIMECs) in the lower channel, enabling the investigation of drug passage and nutrients ingestion across a more natural barrier [216,247].

Therefore, organoids-derived intestine chips are very useful tools to realize clinically relevant human intestine models. A transcriptomic analysis indicated that these human primary cells-based systems more closely emulate the human small intestine scenario compared to Caco-2-based gut-on-a-chip and duodenum biopsy-derived enteroids [216,247]. Kasendra *et al.* [216,247] found that a subset of 305 genes associated with fundamental biological intestine functions (e.g. digestion, metabolism, transport, detoxification) was expressed in their Duodenum Intestine-Chip similarly to the duodenal native tissue, highlighting the potential of this platform to investigate biokinetics and biotransformation processes of nutrients and drugs (as illustrated in Fig. 4F). As matter of fact, the apical localization and function of the major drug transporters, such as MDR1, BCRP and PEPT1, and a high expression of the cytochrome CYP450 (CYP3A4) were confirmed.

Microfluidic devices thus offer the possibility to *in vitro* thoroughly reproduce the biochemical processes that dynamically occur in the human intestine. Clearly, the synergistic use of primary cells and fluidic chips allows the establishment of physiologically relevant pre-clinical platforms for better predictions of human PK, toxicity risks as well as an improved *in vitro*-to-*in vivo* data extrapolation compared to immortalized cell lines-based chips.

Unfortunately, to date, some technical limitations persist in these systems, such as fluid leakages, pump requirements, difficulties in performing robust and high-throughput experiments [249]. Moreover, even though PDMS holds advantageous properties, it can adsorb small and hydrophobic molecules, influencing PK and PD evaluations [250]. Therefore, there remains a compelling need to further improve intestinal OOC systems for increasing their robustness and reliability as drugs screening platforms (Table 4). Alternative manufacturing techniques have already been adopted to realize other types of micro-physiological systems for drug delivery and toxicity assays [251–253]. An innovative research was recently published by Marrella *et al.* to *in vitro* elucidate the absorption mechanisms of different sugars (mannitol and lactulose) in healthy and pathological conditions by incorporating the Epi-Intestinal tissue within a commercial fluidic device (named MIVO®), presented in Fig. 4G, capable to resemble the physiological stimuli of the intestinal

Table 5
Summary of model drugs permeation throughout the intestinal epithelium *in vitro* models. For Caco-2 model, the median of the P_{app} data set considered from literature was selected as representative value to be reported, as shown in Fig. SI-1.

				Papp ($x10^{-6}$ cm/s)										
	pKA	BCS Class	Fa	PAMPA	PVPA	CACO-2	TC-7	MDCK	MDCK-MDR	HT29-MTX	HIEC	HT-29-MTX/ CACO-2 10%	HT-29-MTX/ CACO-2/ RAJI	3D Villi CACO-2/HT-29-MTX	EPI-INTESTINAL	CACO-2 on chip
Atenolol	9,6	III	50	2,06	0,22	0,47	0,21	0,58	0,13	5,93	0,68	0,2	16	-	0,6	60
Acetaminophen	9,9	I	85	2,39	7,3	31,9	9,2	35	-	-	-	-	-	-	-	28
Amoxicillin	2,4	III	45	5,3	-	0,33	0,32	0,24	-	-	-	-	-	-	0,6	0,58
Antipyrine	2,2	I	100	9,12	18,6	47,23	50,5	150	19,35	37,6	33	17,82	-	12,3	-	950
Caffeine	14	I	98	12,54	4,32	26,3	21,8	79,3	20	30,5	-	24,38	55,7	13,67	-	3450
Carbamazepine	15,96	II	100	53	18,6	50,3	17,01	-	19,62	-	62	18,74	36,4	13,65	19,7	150
Cimetidine	6,8	III	60	2,8	0,89	0,74	1,86	1,04	0,97	0,86	1,4	-	24,3	-	1,9	20
Furosemide	4,7	IV	40	3,6	1,41	0,29	-	0,62	1,97	5,6	-	1,03	12,4	8,55	0,7	110
Hydrochlorotiazide	7	IV	55	1,02	0,51	0,51	-	1	-	-	3,3	7,58	-	6,56	-	270
Ketoprofen	3,88	II	100	33,8	5,51	24,36	61	20	-	21	-	16,93	-	14,6	-	5,9
Metoprolol	9,7	I	95	7,8	3,23	26,95	21,6	150	-	22,13	16	22,77	32,3	-	8,4	-
Naproxen	4,19	II	100	49,5	3,79	53,07	89,9	-	-	-	-	17,88	45,7	15,26	15,5	1080
Propranolol	9,5	I	97	36,1	3,41	21,8	21,65	170	34,19	8,45	22	13,38	-	14,07	8,4	390
Ranitidine	8,2	III	52	4,13	0,4	0,49	0,68	-	0,95	-	0,89	7,63	-	9,02	1,1	-
Salicylic Acid	3	I	100	0,59	0,59	15,8	3,55	10	-	2,61	-	-	-	-	-	-
Terbutaline	10,1	III	40	0,23	0,64	0,47	0,128	1	-	6,37	0,7	-	-	-	-	-
Verapamil	8,92	I	100	38,4	9,3	17,2	2,98	25,6	15,2	-	-	15,48	-	11,73	5,7	5,2
Ref	[255,256]	[256,257]	[255,258,259]	[74,260–263]	[64,76,264–267]	[115,155,268–275]	[57,113,115,134,276]	[255,277,278]	[278–280]	[155,260]	[150,151]	[155,190]	[281]	[190]	[85,219,221]	[56,233,282,283]

environment [219].

As a whole, these dynamic platforms hold enormous potential to study intestinal activity in a living human-mimicking environment by imposing luminal and basal flow as well as recreating peristalsis-like motions. Several improvements may be further carried on, for example by considering the spatial variations which exist along the intestine, simulating the different segments of this organ within the same *in vitro* model. Furthermore, the integration of the gut microbiota and immune cells could open up new avenues for better reproducing the *in vivo* system, boosting the growth and the quality of more reliable *in vitro* options, accordingly to the 3R (Reduce, Refine, Replace) principles [254].

6. Discussion and conclusions

It is commonly recognized that ADME properties are crucial to a drug candidate clinical success. To date, poor oral bioavailability - due to inefficient intestinal absorption - has been identified as the primary cause of the high rate of new drug approval failures.

For this reason, several *in vitro* culture systems mimicking the intestinal epithelium have largely spread in the early stages of drug discovery and development to more rapidly predict intestinal permeability and simultaneously reduce animal testing, thus accelerating pharmaceutics translation into clinics. Table 5 shows P_{app} values obtained from different studies and laboratories by adopting the intestinal epithelium *in vitro* models previously described, focusing on 17 model drugs that belong to different BCS class and are characterized by various pKA.

2D models are currently the most standardized platforms since they allow for cost-effective and high-throughput screenings. In particular, Caco-2 cells have been accepted as a gold standard due to their capability to closely resemble the enterocytic phenotype. In fact, Caco-2 and TC-7 cellular models displayed a much higher correlation with *in vivo* data if compared to synthetic models (Fig. SI-2). However, while these conventional cell cultures demonstrated good quantitative correlations with absorbed fraction in humans for drugs transcellularly transported, solely good qualitative results were achieved for other routes, due to their colonic origin and the altered expression of fundamental metabolizing and transporting proteins. Nevertheless, the co-culture of Caco-2 and HT-29-MTX cells - with or without the presence of a 3D scaffold displayed an improved correlation with *in vivo* data (R^2), as depicted in Fig. SI-2.

Hence, with the increasing evidence that 3D multicellular *in vitro* models better recapitulate the *in vivo* environment, several groups have developed more predictive tools for studying xenobiotics ingestion and digestion through the intestinal barrier. Among them, organoids are capable of reproducing multilineage differentiation as well as the 3D morphology of native tissue; nevertheless, their use is limited in this field because of the inaccessible luminal surface. As a result, easier-to-use human reconstructed intestinal tissues have been commercialized to perform bidirectional permeation studies from the lumen to the bloodstream and vice versa. The relationship between P_{app} and Fa was found to be higher than 0.98 by using the Epi-Intestinal^{TM} tissue, where multiple cells are present.

Anyhow, the lack of dynamic stimuli that mediate ADME processes in the human body prompted the manufacture of micro-physiological technologies capable of emulating chemical gradients, luminal flow and peristaltic movements in a highly controllable manner. Tremendous progress has been recently obtained by coupling organotypic grafts with fluid-dynamic systems in the GIT research. However, many challenges to gain reproducible assays need to be faced by using these platforms. Indeed, a systematic characterization of drugs, standardized protocols for culturing epithelial and organoid-derived cells, as well as a clear metrics of choice of manufacturing techniques and materials are lagging behind to translate such devices to the market. In light of this, a plethora of efforts will have to be done to improve the experimental reproducibility between and within laboratories and thus the *in vitro* to *in vivo*

data extrapolation, finally obtaining a robust data set through which the reliability of these models could be demonstrated.

Accordingly, the future implementation of these aspects, together with the realization of more *in vivo*-like models that should include the entire microbiome, a mucus layer and other cell types (e.g. immune cells), will increase the robustness and the predictive potential of these systems.

Author contributions

A. Fedi conceptualized the work; A. Fedi, C. Vitale and G. Ponschin performed data curation; A. Fedi, C. Vitale and G. Ponschin wrote the original draft; A. Fedi, C. Vitale, S. Ayehunie, S. Scaglione revised and edited the original draft; M. Fato and S. Scaglione supervised the work; S. Scaglione revised the final manuscript. All the authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.05.028.

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