A Shared Perspective on in Vitro and in Vivo in Models to Assay Intestinal Transepithelial Transport of Food Compounds

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ABSTRACT: Assessing nutrient bioavailability is complex, as the process involves multiple digestion steps, several cellular environments, and regulatory-metabolic mechanisms. Several *in vitro* models of different physiological relevance are used to study nutrient absorption, providing significant challenges in data evaluation. However, such *in vitro* models are needed for mechanistic studies as well as to screen for biological functionality of the food structures designed. This collaborative work aims to put into perspective the wide-range of models to assay the permeability of food compounds considering the particular nature of the different molecules, and, where possible, *in vivo* data are provided for comparison.

KEYWORDS: *intestines, transepithelial transport, nutrient bioavailability, food compounds*

1. INTRODUCTION

To assess the impact on human health, it is necessary to evaluate the bioaccessibility and bioavailability of compounds in food such as bioactives, macro- and micronutrients, allergens, and contaminants with either positive or negative effects on health. This information underpins the cause-and-effect relationship of bioactive compounds, identifies the minimum intake of essential micronutrients, and establishes safe doses of contaminants or allergens. The models used for this purpose typically adhere to the general guidelines used in assessing drug bioavailability. Absorption, distribution, metabolism, and excretion (ADME) (with potential toxicity) are the key biological processes involved in drug, nutrient, and molecule's pharmacokinetics. These compounds should escape intestinal metabolism in the lumen and gut wall, in order to pass through the epithelial cell layer, to enter the portal vein, and to reach the liver and ultimately the systemic circulation.[1] It has also been shown that food-derived substances and the structures formed during digestion are able to modulate the intestinal absorption functions by themselves,[2] which adds an extra complexity to the food-intestinal epithelium-absorption equation. The food matrix will also contribute to or undermine compound survival. Along with studies in humans, digestion and absorption models are needed to understand the mechanisms beyond oral delivery, making it possible to systematically study an increasing number of molecules of interest and their interactions. The present work aims to put into perspective the wide-range of models used to assay the permeability of food compounds by taking into consideration the particular nature of the different molecules, and, where possible, comparison with *in vivo* data. This work relies on the experience of a consortium of researchers with activities focused on intestinal absorption models.

2. IMPACT OF THE PROPERTIES OF FOOD-DERIVED COMPOUNDS ON THEIR TRANSPORT THROUGH THE INTESTINAL EPITHELIUM

The intrinsic characteristics of the ingested compounds and the actual form in which they reach the intestinal epithelium determine the mechanism to be absorbed, and the transport model should ideally mimic this. Figure 1 outlines the major transport mechanisms for macronutrients.

2.1. Transport of Lipids. Dietary lipids consist of a wide array of chemically diverse polar and nonpolar lipids. Triacylglycerols (TAG) are the most abundant lipids, accounting for 90−95% of the total energy provided by dietary fat, which also includes phospholipids, sterols (e.g., cholesterol and β-sitosterol), and other compounds like lipophilic vitamins. Pancreatic lipases in the small intestine hydrolyze TAG into diacylglycerols, monoacylglycerols, and free fatty acids (FFAs). FFAs are then incorporated into spontaneously forming mixed vesicles (or dietary mixed vesicles), which shuttle lipophilic compounds through the "unstirred water layer" to reach the enterocyte brush border. At the brush border, the mixed vesicles disperse and unesterified

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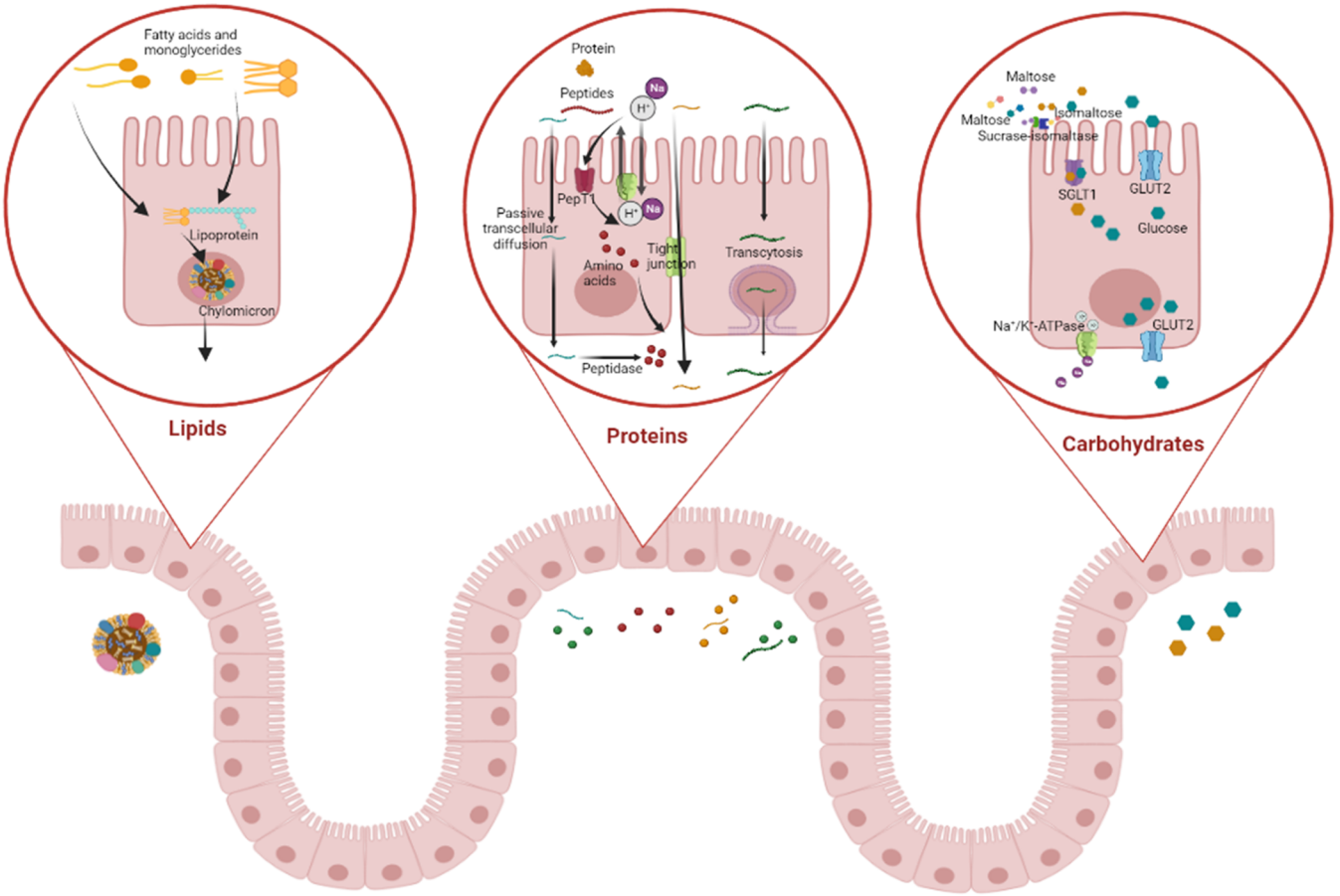

Figure 1. Outline of the specific transport mechanisms described for macronutrients through the intestinal epithelium.

FFAs cross passively through the membrane into enterocytes, where they are immediately re-esterified to form TAGs. TAGs are then packaged into chylomicrons, which are initially secreted into intestinal lymph vessels as they are too large for blood capillaries. Long chain FFAs can be actively taken up by enterocytes and are also esterified into TAGs before incorporation into chylomicrons, while short chain FFA are sufficiently hydrophilic to be transported directly to the blood circulation.[3] Intracellular assembly of TAG-rich chylomicrons requires predominantly apolipoprotein B-48 biosynthesis and subsequent cotranslational lipidation with TAGs.[4] Lipid bioavailability in humans is dependent on the initial emulsification (and thus droplet size) of dietary lipids to allow for lipase to digest TAGs into FFAs and the subsequent incorporation into mixed vesicles who shuttle digested lipids to the enterocyte brush border.

The intestinal transport of lipids *in vitro* necessitates the prior formation of mixed micelles, a step accomplished through relevant gastrointestinal simulations such as the INFOGEST method. However, when conducting studies directly involving lipids, it is imperative to prepare the mixed micelles beforehand. At the cellular level, the most crucial factor of an *in vitro* model for lipids is their ability to form and secrete lipoproteins. In addition, the expression of fatty acid binding proteins is relevant to enterocytes functionality in studies involved in lipid transport and metabolism. The human immortalized Caco-2 cell line, once polarized and differentiated to monolayers, is well capable of lipoprotein production and secretion,[5] which are the largest and most efficient lipoprotein lipid transporters. Caco-2 cells have been compared to human colonic explants for their ability to esterify lipids, synthesize apolipoproteins, and assemble lipoproteins.[6] Differentiated Caco-2 cells, like enterocytes, incorporate oleic acid into cellular phospholipids, TAGs and cholesterol esters, however with limited capacity to export newly synthesized lipids. Depriving Caco-2 cells of fetal bovine serum in the apical medium compartment during differentiation for one to two weeks prior to oleic acid treatment, was found to improve chylomicron production.[7] Beyond the standard Caco-2 models, cocultures with mucus secreting cells, Caco-2/HT29-MTX, demonstrate a higher functionality for oleic acid uptake, storage, and subsequent basal lipid release versus monoculture Caco-2 barriers.[8] Salvini et al. have shown that Caco-2/TC7 cells (a subclone of Caco-2) exhibit a higher rate of secretion of oleic acid (18:1) than the parental Caco-2 cells, with 86% of net apical uptake which compared well to 97% observed in human subjects.[9]

2.2. Transport of Amino Acids, Peptides, and Proteins. In the healthy gut, dietary proteins are hydrolyzed to a different extent into peptides and amino acids according to their natural or processing-derived susceptibility to proteases. It is classically accepted that 90% of absorbed dietary proteins are represented in the circulation by amino acids and 10% as dipeptides and tripeptides. However, food-derived peptides over this number of amino acids have been also identified in plasma in animals and in humans following dedicated extraction and up-to-date peptidomic tools.[10,11] In the human gut, amino acids have a number of transport systems

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involved in the absorption from the lumen into the enterocytes that vary in solute specificity and may present an electroneutral or electrogenic transport process. These include $b^{0,+}$AT, rBAT, CAT1, CAT2, y $^+$LAT1, and y$^+$LAT2 for basic amino acids, B^0AT and LAT1 for neutral amino acids, and EAAT1 and EAAT3 for anionic amino acids.[12] The basolateral membrane of the enterocytes also expresses at least six different Na^+-dependent and -independent amino acid transport systems that mediate the exit to the blood. These latter transport systems may work in either direction depending on the luminal amino acid concentration and cellular demand. Quantitatively, the absorption of peptides across the intestinal lumen is the predominant mode of absorption of protein digestion products. Peptides in the intestinal lumen can be transported through the intestinal epithelium into the bloodstream via a paracelullar route, transcytosis, passive transcellular diffusion, and/or peptide transporters. Di- and tripeptides are efficiently transported by proton gradient cotransport. The solute carrier 15 (SLC15) family of transporters are H^+-dependent transporters involved in the uptake of di- and tripeptides in the cells. Within this family PEPT1 (*SLC15A1*) and PEPT2 (*SLC15A2*) mediate intestinal absorption of di- and tripeptides, including anionic and cationic peptides, as well as different peptide drugs. In PEPT1, transport efficiency depends on the net charge of the peptides and the relationship between membrane potential and external pH, with the transport of neutral and negatively charged dipeptides being stimulated by lowering luminal pH, while an increasing pH is coupled with transport of positively charged peptides.[13] Other authors have reported that PEPT1 is unable to bind peptides with high positive charge, such as KK or KWK.[14]

In addition to peptide transport, several human studies have confirmed that small amounts of intact proteins, such as cow's milk β-lactoglobulin and hen's egg ovalbumin, can be found in the plasma of human volunteers following their consumption.[15] The mechanisms by which these proteins with allergenic potential are transported across the intestinal epithelial barrier include paracellular or transcellular transport. These transport mechanisms depend on a number of factors, including intrinsic protein properties, the physiological state of the intestine, as well as extrinsic, food-related factors.[16] Moreover, although they have been always thought to be purely secretory cells, small intestinal globlet cells can take up luminal antigen and deliver it to lamina propria dendritic cells.[17]

LAT1 and LAT2 transporter transcripts and proteins are present in Caco-2, with LAT2 playing an important role in amino acid transport. In contrast with B^0 and y+ systems, which have been reported to be downregulated when Caco-2 cells stop proliferating, L-glutamate transport capacity was found to increase in differentiated cells compared to that found during proliferation, and this increase was correlated with the level of EAAT1 mRNA.[18] Caco-2 cell monolayers express apical and basolateral peptide transporters of the SLC15 and cadherin gene families. Although the Caco-2 model has been proven to be versatile for peptides, the gene expression of SLC15A1 (PEPT1), SLC15A3 (PHT2), SLC15A4 (PHT1), and CDH17 (human peptide transporter 1, HPT-1) has been described to be increased compared to the human gastrointestinal tract,[19] which may overestimate *in vitro* results. However, culture length, cell density, or cell supplier may influence peptide transporter expression levels as it has been evidenced in interlaboratory comparisons. The effect of culture conditions has also been evidenced in HT-29 cells, where culturing in galactose-rich medium favored the expression of peptide transporters PEPT1, PEPT2, PHT1, and PHT2.[20] Although HT-29/MTX cells are capable of transporting phenylalanine at a lower rate than Caco-2, and the dipeptide (Gly-Pro) at rates similar to that of Caco-2, the transporters need to be specified.

To elucidate the mechanisms of allergen transport, studies have heavily relied on human intestinal transport models, with the Caco-2 model being the most frequently used. For example, the Caco-2 model was used to show that some, but not all allergens (e.g., wheat α-gliadins, peanut allergens, and the shrimp allergen Pen j 1), increase the Caco-2 monolayer permeability and promote their own paracellular passage.[21] Similarly, the Caco-2 model was used to demonstrate that some allergens, including cow's milk β-lactoglobulin or peanut Ara h 1 and Ara h 2, are transported across the intestinal epithelial barrier by a transcellular route during which they are partially degraded.[21,22] An *in vivo* matured, intestinal organoid-based model of human induced pluripotent stem cell (iPSC) was used to demonstrate that cow milk allergens could be transported through goblet cells and secretory antigen passages in humans.[23] The relevance of these mechanistic data for protein and oligopeptide absorption *in vivo* remains to be confirmed using human studies, where possible, preferably by comparing nonallergic and allergic individuals.

2.3. Transport of Carbohydrates. Most consumed carbohydrates in adulthood are sugars and starches. Sugars include monosaccharides (e.g., glucose, galactose, and fructose) and disaccharides (e.g., lactose, sucrose, and others) which are hydrolyzed by the sucrose-isomaltase and maltase-glucoamylase brush border enzymes within the small intestine. Salivary and pancreatic amylases are responsible for the initial breaking down of nonresistant starch to oligosaccharides, followed by further digestion by small intestinal brush border enzymes (i.e., sucrase, sucrase-isomaltase complex, β-glycosidase complex, trehalase),[24] which are responsible for the final cleavage into individual sugars. In the small intestine, the monosaccharides are then taken up by the enterocytes through specific transporters. Sodium-glucose linked transporters, SGLT1, are mainly expressed in the upper third of the small intestinal villi, driving the transport of the monosaccharides D-glucose and D-galactose. SGLT1 uses the sodium (Na^+) gradient created by the basolateral Na^+/K^+-ATPases to take up the monosaccharides into the enterocytes. While SGLT1 is localized at the brush border membrane of the enterocytes, glucose transporter 2 (GLUT2) is thought to be localized to the basolateral membrane and only recruited into the apical membrane under high luminal glucose presence.[25] Na^+-independent GLUT mediates passive transport, such as fructose transport by the uniporter GLUT5. It is currently accepted that also polysaccharides such as hyaluronan, glucan, or pectin can be absorbed from the intestine by active transport and passive diffusion processes. However, although they have been detected in the intestine, they do not easily reach the systemic circulation, as it was shown for glucan and pectin.[26] Charge, molecular weight, spatial structure, and dose are the main determinants of the transported amount. In addition, they can be taken up by immunosurveillant microfold (M) cells and then distributed to the surrounding mucosal immune system in Peyer's patches.

Studies showed that SGLT1 has a lower level of expression in Caco-2 cells compared to human enterocytes, and this varies

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between laboratories[27] rendering it difficult to compare with *in vivo* studies. On the other hand, the presence of GLUT2 in the apical membrane of the differentiated enterocytes and its contribution to glucose absorption, is controversial. This has led to some authors to consider the Caco-2 cell monolayers as a poor model for studying intestinal glucose transport, at least the SGLT1-mediated transport.[28]

2.4. Transport of Micronutrients. It has been long believed that intestinal absorption of vitamins and minerals was mostly a passive process, and lipophilic vitamins were absorbed together with digested lipids and hydrophilic vitamins with water. However, carrier mediated absorption is now well established for most hydrophilic vitamins (ascorbate, biotin, folate, niacin, pantothenic acid, pyridoxine, riboflavin, and thiamin).[29] Similarly, many minerals are absorbed by the involvement of carrier proteins or specific receptors to control uptake and avoid cytotoxicity (e.g., haem iron). Lipophilic micronutrients are best absorbed when lipids are coingested to trigger a postprandial response; however, there is uncertainty as to what lipid dose is optimal. Carrier systems for the absorption of vitamins A, E, K1, and carotenoids have also been identified while vitamins D and K2 seem to be absorbed via a passive mechanism.[30] The bioacessibility of lipophilic vitamins in different types of foods is commonly measured by their ability to assemble within micelles during digestion experiments. It is assumed that once they are in the micelles, they will be absorbed.[31]

2.5. Transport of Polyphenols. Polyphenols are secondary metabolites of plants, with the natural biological function to protect against external factors such as the sun's rays or the infection by microorganisms. The bioavailability of polyphenols is low.[32] Although the aglycones can be absorbed from the small intestine, most dietary polyphenols are in the form of esters, glycosides, or polymers that cannot be directly absorbed. These must be hydrolyzed by intestinal enzymes or by the colonic microbiota, with, for example, procyanidin monomers, dimers, and rarely trimers detected in the plasma. Polyphenols are metabolized and conjugated in the intestinal cells and later in the liver by methylation, sulfation, and/or glucuronidation, or return to the digestive tract through the enterohepatic circulation. In the large intestine, the intestinal nonabsorbed polyphenols and conjugated polyphenols are catabolized to ring-fission products (i.e., phenolic acids) by the enterobacteria. These metabolites can then in turn impact the intestinal immune function.[33] Hence, the biotransformation of polyphenols and the generated metabolites is a key point in studying the bioavailability. In fact, it has been pointed out that studies on the mechanisms of the biological effects of polyphenols on human cell lines should use the actual metabolites produced *in vivo*, that is the conjugates of the original aglycones or gut microbiota metabolites. *In vitro* studies have some limitations on reproducing the human biotransformation enzymes. Janssen et al.[34] compared the expression of cytochrome P450 enzymes in Caco-2 monolayers with human intestinal organoids derived from human iPSC. Caco-2 showed lower gene expression and activity of CYP3A4 than organoids, but a better expression of other enzymes such as CYP1B1, CES1, SULT2A1, and UGT1A8. Still, organoids are considered to be preferable for biotransformation studies.

To impart their biological activity, it is important that these phenolic compounds be released from the food matrix and become bioaccessible. Of particular interest is the effect of processing modifications on the bioavailability of these compounds, and many works have dealt with the means to improve their bioaccessibility, for example, by using vesicles as delivery systems.[35] In the analysis of six dietary polyphenols (caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin), strong correlations have been shown between the experimental partition coefficient and both parallel artificial membrane permeability assays (PAMPA) and Caco-2 cell monolayer permeation data.[36] The results showed that passive diffusion is the main transport mechanism for these compounds, and their poor absorption is consistent with the documented poor oral bioavailability in humans.

2.6. Transport of Food Contaminants. The European Food Safety Agency, EFSA, has assessed and classified chemicals that can contaminate food into several groups, including natural toxins like mycotoxins, alkaloids and bacterial toxins, environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), metals, and process contaminants such as acrylamide, chloropropanols, and heterocyclic aromatic amines (HAA) (https://www.efsa.europa.eu/en/topics/topic/chemical-contaminants-food-feed). Moreover, new concerns are emerging, such as microplastics contaminating food. Little information is available on the pharmacokinetics of microplastics, which are prone to produce secondary products and can appear as particles of different sizes.[37]

The gastrointestinal mucosa is the first barrier that these compounds encounter before entering the systemic circulation. If bioaccessible, most of these chemical groups are transported through the transcellular pathway and less through the paracellular way. The transcellular barrier is composed of the surface cell membranes, which has a much larger surface area than the surface of tight junctions (99.9 versus 0.01%), where paracellular transport occurs.[38] Therefore, compounds using the transcellular pathway (e.g., PAHs, PCBs, pesticides, HAAs, heavy metals) tend to have a higher absorption area available than compounds that cross the epithelium via the paracellular pathway (e.g., some mycotoxins and chloropropanols). However, the absorption pathway alone does not determine the compound levels and bioavailability. The measurement of apparent permeability (P_{app}), which quantifies the flux of a compound through a membrane while normalizing for the membrane surface area and donor concentration, serves as an objective method for expressing the absorption of toxicants. High permeability is indicated by values greater than 1×10^{-6} cm/s. This threshold is exceeded in the case of mycotoxins, with compounds as Aflatoxin B1, showing remarkable absorption (78×10^{-6} cm/s)[39] or chloropropanols (30×10^{-6} cm/s) where free but not esterified forms are able to cross the monolayer.[40] Unfortunately, with a few exceptions, these values are largely absent from the existing literature, probably due to the high number of sampling settings needed. Consequently, future investigations should focus on generating *P*app values for more toxicants.

3. THE CHALLENGE TO DETERMINE *IN VIVO* ABSORPTION RATES

There are many challenges in the determination of absolute food compounds bioavailability. To determine nutrient bioavailability in humans, simple measurement of nutrient appearance in plasma is not sufficient as many nutrients are tightly regulated and metabolized, and newly absorbed compounds need to be separated from those already present

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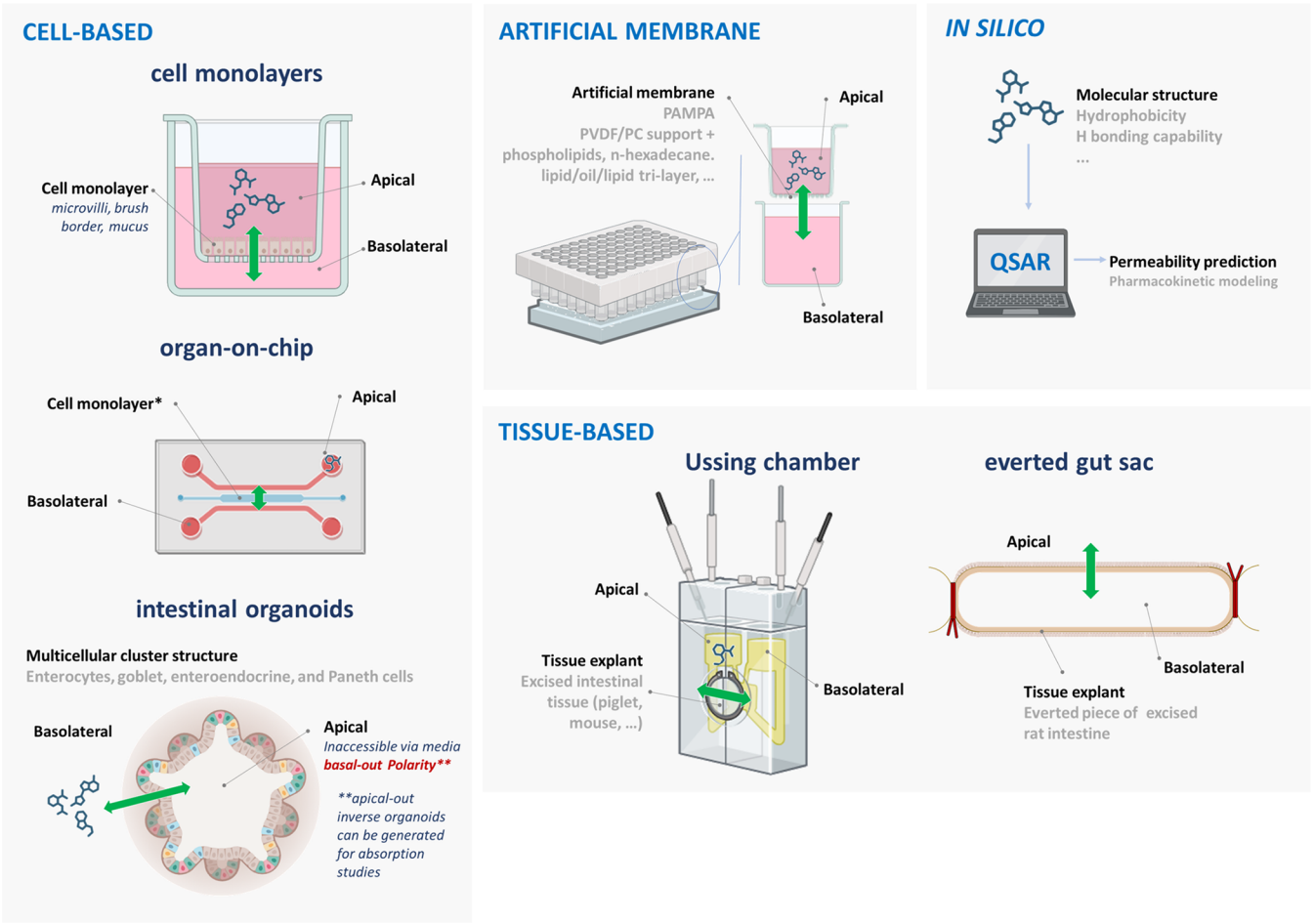

Figure 2. Graphical overview of the main models used for transport studies. Apical compartment: intestinal lumen, mucosa, donor; basolateral compartment: systemic circulation, serosa, acceptor). Green arrow indicates the absorption/secretion direction.

in the body. This difficulty has been highlighted by *in vivo* absorption studies with different food components.

The "chylomicron fraction method" separates recently absorbed lipophilic micronutrients. Immediately after absorption in the enterocytes, newly absorbed lipophilic nutrients are indeed "packaged" into chylomicrons, while whole blood contains both previously absorbed and recently absorbed nutrients. Chylomicrons can be isolated from fresh plasma by density gradient ultracentrifugation, and the compound of interest can be then quantified by HPLC or LC-MS, although the method is not valid for hydrophilic micronutrients. Research into the roles of endogenous versus dietary fat has been hindered because of the difficulty in separating dietary fat transported from the intestine in chylomicrons and fat transported from the liver in VLDL by ultracentrifugation. An alternative to the ultracentrifugation is the prediction of chylomicron triglycerides in human plasma using metabolomics tools such as nuclear magnetic resonance and chemometrics.[41] Alternatively, chylomicrons may be purified from VLDL in postprandial human plasma samples by an immunoaffinity method. This method improved the evaluation of the relative contribution of the small intestine versus the liver to postprandial lipid fluxes. In addition, to address the dietary influences of postprandial lipids on cardiovascular risk, this method coupled to a tracer feeding study (^{13}C acetate) generated preliminary evidence suggesting enteral conversion of dietary sugar to fat via *de novo* lipogenesis.[42]

These methods, however, result in values of relative bioavailability and not absolute bioavailability. Absolute bioavailability is the total amount of a compound taken up from the gut into the blood and is usually determined in animals as both an oral and an intravenous dose is required. This term might strictly include the fraction metabolized by cells, like amino acids used by cells as an energy source. The use of stable isotopes is a valid alternative to measure bioavailability and is well suited to study hydrophilic compounds. However, it is technically challenging and expensive. This strategy was used to determine the intact absorption of dipeptides in rats.[43] On a rather different tack, to determine protein digestibility, a dual-stable-isotope method was developed, where the ingestion of an intrinsically ^{2}H-labeled test protein along with C^{13} labeled standard protein of known digestibility permits an accurate measure of digestion and absorption to meet human protein requirements.[44]

The body metabolism is a critical factor in measuring nutrient bioavailability. For example, many studies have attempted to measure the bioavailability of bioactive peptides. In a pharmacokinetic study in rats, the transport and distribution of a digestion-resistant casein-derived antihypertensive hexapeptide, HLPLP, showed an absorption rate of 5.2%. The same compound had previously shown a transepithelial flux in the Caco-2 model of less than 1‰, in accordance with the already reported lower absorption values in this cell model. More importantly, the absorption *in vivo* was

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Table 1. Advantages and Disadvantages of Different Cell-Based and Tissue-Based Intestinal Models, As Well As Several Common Alternative Intestinal Models Used for Transport Studies[a]

	Intestinal model	Origin	Easy to culture	Costs	Availability	Cell types	Differentiation	Maturity	Main advantages	Main disadvantages
Cell lines	Caco-2	Human (white male, 72y, colon adenocarcinoma)	Yes	Cheap	ATCC/ECACC	Enterocytes	16-21 days	Mature	• Easy to culture • Cheap • Quite reproducible • Commonly used for transport studies	• Only enterocytes • No mucus layer • Derived from colon carcinoma • Physiologically not fully comparable to human intestine • Length of differentiation • Very tight epithelial layer
	HT29	Human (white female, 44y, colon adenocarcinoma)	Yes	Cheap	ATCC /ECACC	Enterocytes and a small proportion (<5%) of goblet-like cells	30 days	Mature	• Easy to culture • Cheap	• Only enterocytes • Derived from colon carcinoma • Length of differentiation • No barrier integrity
	Caco-2/ HT29-MTX	Human (co-culture of Caco-2 with a subclone of HT29 with significant mucus production)	Yes	Cheap	ATCC/ECACC	Enterocytes and mucus-producing goblet-like cells.	14-21 days	Mature	• Easy to culture • Cheap • Mucus layer	• Limited number of cell types • Derived from colon carcinoma • Physiologically not fully comparable to human intestine • Length of differentiation
	Caco-2/HT29-MTX/Raji-B	Human (co-culture of Caco-2, HT29-MTX, and Raji-B (black male, 11y, burkitt's lymphoma))	Medium	Cheap	ATCC/ECACC	Enterocytes, mucus-producing goblet-like cells, and M-like cells	21 days; addition of Raji-B at day 14	Mature	• Easy to culture • Cheap • Multiple cell types	• Limited number of cell types • Artificial co-culture for which the physiological representativity remains to be validated • No clear, defined protocol of differentiation
Tissue	Ussing Chambers	Human, different origins	Medium	Medium	Local sources (clinical)	Identical to location of biopsy	None	Mature	• Multiple cell types • Regional differences • Mature • Easy access to lumen or basolateral side • No cell culture infrastructure needed	• Material difficult to obtain (clinical protocol needed) • Material from diseased individual? • Not high-throughput • Interindividual variability • Short viability (150 min max)
	Ussing Chambers	Pig, mouse, rat			Local sources					• Non-human material • Medium- to low-throughput • Short viability (150 min max) • Source needs to be sacrificed
	Everted Sac	Mostly rat	Yes	Medium	Local sources	Identical to location of biopsy: mostly sections of small intestine (duodenum, jejunum, ileum) and/or colon.	None	Mature	• Multiple cell types • Regional differences • Mature • Easy access to lumen or basolateral side • No cell culture infrastructure needed	• Non-human material (mostly rat) • Not high-throughput • Short viability (150 min max) • Source needs to be sacrificed
Primary	iPSC organoids (2D and 3D)	Human, different origins	No	Expensive	Local sources or cell repositories	Enterocytes, enteroendocrine cells, Paneth cells, some goblet cells, mesenchymal cells	28-60 days	Immature (3D) or foetal (2D) phenotype	• Multiple cell types • Intestinal localization can be skewed with cytokines • Presence of mesenchymal cells	• Expensive • Challenging to access to lumen or basolateral side • Immature (3D) or foetal (2D) phenotype • Interindividual variability • Length of differentiation
	ASC organoids (2D and 3D)	Human, different origins	No	Expensive	Local sources or cell repositories	Dependent on the differentiation cocktail: enterocytes, enteroendocrine cells, goblet cells, Paneth cells	5-7 days (3D) or 3-5 days (2D), immature organoids can be passaged	Mature	• Multiple cell types • Different intestinal locations can be mimicked • Possibility to skew differentiation towards different cell types	• Expensive • Difficult to access lumen or basolateral side • Material difficult to obtain • Interindividual variability
	Organotypic small intestinal models (e.g. MatTek™)	Human, different origins	Medium	Expensive	MatTek™	Majority of intestinal epithelial cells present. Possibility to add mesenchymal cells.	2 weeks	Mature	• Multiple cell types • Mature • Easy access to lumen or basolateral side • Relatively high throughput	• Expensive • Commercial solution • Donor variability
Synthetic	Artificial membrane	N/A	Yes	Very cheap	Commercial	N/A	N/A	N/A	• Easy to use & easy access to lumen or basolateral side • Cheap • Possibility to work with different porosities, materials and geometries • Screening tool. • No cell culture infrastructure needed	• Absence of intestinal cell monolayer • Only passive transport studied, no paracellular or active transport • Membrane composition does not resemble biological membrane

[a]iPSC, inducible pluripotent stem cell; ASC, adult stem cell; N/A, not applicable.

followed by metabolization of the peptide into at least three species (HLPL, LPLP, and HLP). This highlights the difficulty in relating bioavailability to bioactivity, as these biotransformed peptides could elicit as well antihypertensive activity.[45] The plasma concentration of similar antihypertensive peptides in humans was on the order of pico-or nanomolar, representing an absorption rate below 0.1%. In another study, MALDI-MS imaging was conducted to visualize the antiatherosclerotic dipeptide WH in the rat intestinal membrane as compared with the reverse sequence HW. Whereas the first sequence was absorbed intact in blood, the dipeptide HW was not, despite similar P_{app} values for both peptides in Caco-2 monolayers. The MS imaging approach allowed the hydrolysis of HW by brush border peptidases at the intestinal membrane. These examples support the need to improve the models to evaluate *in vitro* permeability.[46]

In summary, there is no perfect method available for the determination of the absolute food nutrient bioavailability. Human studies are costly and invasive and still generate information only on relative bioavailability. In addition, validated methods are required to collect accurate data from these studies. Correlation of *in vitro* data to *in vivo* findings is also urgently needed.

4. MODELS USED IN NUTRITION FOR TESTING INTESTINAL TRANSEPITHELIAL TRANSPORT

The human intestinal barrier consists of different cell types, including enterocytes, goblet, Paneth, tuft, enteroendocrine, and stem cells. They are present in different proportions and densities from proximal to distal intestine, fulfilling not only a physical separation function but also permitting selective sensing and absorption of nutrients, as well as playing a role in intestinal immunological functions. The transport models are intended to mimic the natural epithelial polarity, i.e., presence of an apical or mucosal side and a basolateral or serosal side and capability of the known mechanisms: paracellular,

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transcellular, carrier-mediated, or vesicle-mediated (Figure 2). *In vivo*, this barrier crossing will be governed by the compound structure and concentration of the bioaccessible fraction. A view on the different systems used to mimic the transit of nutrients or contaminants across the intestinal epithelium is presented in order of increasing *in vivo* representation. Some advantages and limitations of the intestinal models used for transport studies are listed in Table 1.

4.1. Artificial Membrane and *in Silico* Models. Cell-free artificial barriers respond to the need for a low cost, easy handling, and rapid screening model to predict gastrointestinal transcellular passive permeability. These systems make use of a donor and a receiver/acceptor compartment separated by a barrier of defined pore size, usually a polymeric membrane. The permeability values recorded in drug transport are usually far lower than those from intestinal perfusion experiments; however, they are comparable to experiments where permeability is tested with Caco-2 cell monolayers.[1] *In silico* approaches are also often used to predict the permeability mechanistically. Data from PAMPA assays have been subject to numerous quantitative structure−activity relationship (QSAR) studies, and Volsurf computational approaches where the information from 3D interaction energy grid maps is condensed into 2D molecular descriptors for pharmacokinetic modeling. Thus, hydrogen bonding ability in addition to hydrophobicity at a particular pH have been shown to be significant, for example, in determining variations in PAMPA permeability coefficients of natural flavonolignans.[47] Another *in silico* approach was used to rank olefins by a reactivity simulation study. The reactivity was described by the position of the double bond rather than the number of carbon atoms while in the lipophilicity and permeability analysis, both parameter descriptors depended mainly on the number of carbon atoms; these results were predictive of the absorption in an everted sac model.[48] These computational and membrane models are emerging as alternative methods to animal testing for transcellular passive permeability studies (see for example https://data.jrc.ec.europa.eu/collection/id-0088), but comparison with human absorption needs further attention.

4.2. Cell-Based Models. Various cell-based assays have been adopted to analyze food−-intestine interactions and the absorption of dietary compounds. Even though the Caco-2 intestinal barrier model lacks physiological diversity, this cell line represents enterocytes, which are the most numerous cell types in the intestine. Over a period of 21 days, these cells can differentiate into polarized monolayers of absorptive enterocytes with an apical brush border membrane and microvilli. Caco-2 monolayers provide the simplest system to study absorption by paracellular, transcellular, and carrier mediated absorption. *In vivo*, the gut barrier is protected by a dense mucus layer, which should be penetrated by food molecules as a first stage of absorption. To mimic such a barrier *in vitro*, a mucus layer can be overlaid on the monolayers or Caco-2 cells can be cocultured with HT29-MTX adding mucus-secreting goblet cells. This coculture showed no toxic effect against biorelevant intestinal fluids in terms of viability and lactate dehydrogenase release, suggesting the apical mucus barrier was cell protective.[49] Furthermore, to increase cell representation in the monolayer to track allergens or immunomodulators, immune cells, such as RAW264, PBMCs, Raji B, U937, and THP-1, have been added to Caco-2 or Caco-2/HT29 monolayers. The strong correlation (R^2 = 0.84) between permeability data generated from Caco-2 monolayers and from human trials for drug compounds that utilize transcellular passive transport[50] makes this model reliable to study this permeability mechanism. However, this cellular model is not expected to provide absolute absorption values, and relevance to *in vivo* data in relation to other permeability mechanisms must be determined.

Intestinal organoids, also called "mini-guts", are structures grown from stem cells and consist of organ-specific cell types that self-organize and maintain the main physiological characteristics of the intestinal tissue. In this model, a polarized epithelium with tight junctions is developed and includes enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. They are generated from isolated intestinal tissue of mice or porcine immediately after sacrifice or from humans through surgery biopsy and can be maintained in culture in the laboratory over months or preserved under liquid N_2 for longer storage. Although 3D organoids have apicobasal polarity with their apical membranes located toward the organoid core ("basal-out" polarity), apical surfaces are largely inaccessible when stimuli are applied via the media. However, it has been reported that organoids are porous and that solutions can readily reach the apical membrane. Organoids seeded in a 2D layer in bicameral plate increase the relevance of the monolayer and can be cocultured in the presence of gut-derived fibroblasts to add physiological complexity,[51] which was applied to evaluate the barrier integrity in the presence of shear stress and dynamic flow conditions, physiological characteristics found *in vivo*. Other studies evaluated not only the permeability but also the transporter involved in the process. A competitive assay between different cyclic hexapeptides and the radiolabeled Gly-Sar was performed to screen for transporter-mediated uptake of those peptides.[52] The results showed significantly decreased radiolabeled Gly-Sar uptake, with the candidate peptides being substrates for peptide transporter-mediated uptake in humans. This highlights the suitability of intestinal organoids to screen for transporter-mediated uptake, a process under-appreciated in other cellular systems due to poor transporter expression. Because 3D organoids have their apical membranes toward the organoid core, rendering them inaccessible, the use of nonenzymatically dissociated ("broken up") organoids instead of intact ones could be an option to target basolateral and apical transporters at the same time.[52] Similarly, using organoids with an inverse structure (named xenogeneic-free human intestinal organoids), Inoue et al.[53] tested the barrier permeability and showed that the "inside-out" organoids assimilated glucose, dipeptide, and cholesterol through the specific transporters expressed on the cellular surface. Moreover, similar transporter expression (SGLT1, GLUT2, PEPT1, NPC1L1) was found compared to the human intestine. It is important to note that these intestinal tissue-derived organoids have their limitations, as they only consist of epithelial cells and the mesenchyme, the vasculature, neuronal cells, and the immune system are lacking. Also, the epithelial cell composition is dependent on the cocktail of growth factors and cytokines added during culture, which does not necessarily reflect the *in vivo* epithelial cell composition.

Organ-on-chip (OoC) technology represents a new kind of system useful for understanding the complexity of the human intestine. This technology allows to recapitulated tissue-level physiology and function into biologically based microfluidic *in vitro* devices, also known as microphysiological systems

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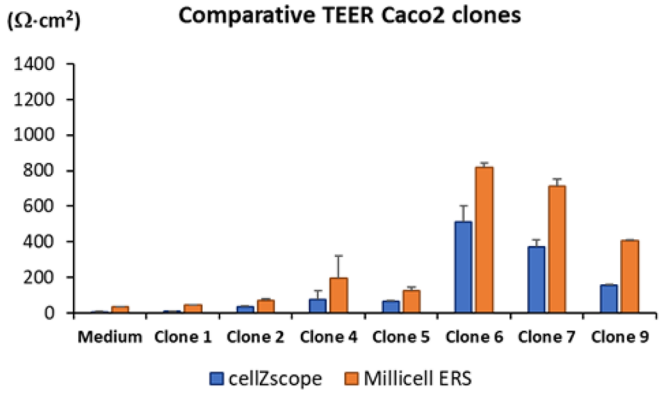

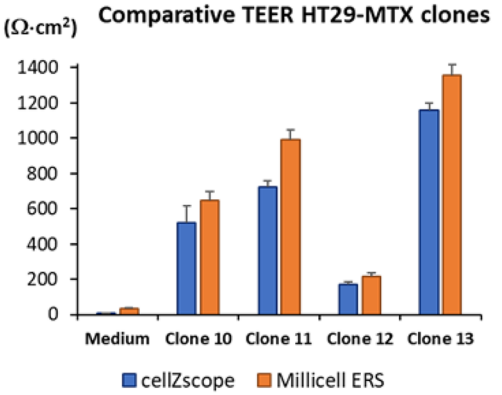

Caco2	Laboratory	Passage	Source
Clone 1	IPLA-CSIC (Spain)	p51	Caco-2 (ECACC-09042001)
Clone 2	IPLA-CSIC (Spain)	p46	Caco-2 (ECACC-86010202)
Clone 4	Nestlé Res. C. (Switzerland)	p50	Caco-2 (ECACC-09042001)
Clone 5	University Porto (Portugal)	p28	Caco-2 (ATCC-HTB-37)
Clone 6	CIAL-CSIC (Spain)	p35	Caco-2 (ATCC-HTB-37)
Clone 7	Teagasc (Ireland)	p23	Caco-2 (ATCC-HTB-37)
Clone 9	UPM (Spain)	p8	Caco-2 (Unknown)

HT29-MTX	Laboratory	Passage	Source
Clone 10	Teagasc (Ireland)	p59	HT29 MTX-E12 (ECACC-12040401)
Clone 11	INSA (Portugal)	p6	HT29 MTX-E12 (ECACC-12040401)
Clone 12	IPLA-CSIC (Spain)	p18	HT29 MTX-6 (provided by T. Lesuffleur)
Clone 13	Nestlé Res. C. (Switzerland)	p54	HT29MTX-E12 (ECACC-12040401)

Figure 3. TEER values of the Caco-2 and HT20-MTX clones from different laboratories analyzed after 21 days of differentiation at IPLA-CSIC by automated impedance-based (CellZscope+) and manual ohmmeter measurement (Millicell ERS).

(MPS). Gut-on-chip systems contain microchannels that are continuously perfused and are covered by different intestinal cell types, arranged to form tissues mimicking the *in vivo* morphology. They try to mimic not only the mechanical, structural, absorptive, and transport properties, but also pathophysiological ones including for instance intestinal microbes or immune cells.[54] Some of the most remarkable advantages of MPS or OoC systems are the peristalsis simulation and flow-induced shear stress. Both biomechanical signals play a fundamental role in cell differentiation. Peristalsis-like strain on cells was reported to induce 3D intestinal villi morphogenesis,[55] while shear stress increased mitochondrial activity or mucus production and therefore drug absorption.[56] Several studies show fully matured intestinal cells after 4 days under dynamic flow instead of a 21 day period for static conditions, so these models could be shortened with the advantage that this entails. However, there are technical needs such as the development of more biocompatible materials, the integration of sensors, and the optimization of microfluidic design; hence, their use in nutrient transport is still at its beginning.

4.3. Tissue-Based Models. Ussing chambers (UC) consist of two halves separated by an epithelium (sheet of intestinal mucosa or a monolayer of epithelial cells grown on permeable supports). In the UC, excised intestinal animal tissue segments are placed between the two halves of the chamber and kept under physiological buffer, gas, and temperature conditions. UC thus may allow investigation into the absorption of several bioactive compounds such as dietary peptides that cannot easily be done *in vivo*. The presence of plasma proteases that further hydrolyze peptides reaching the bloodstream makes the untargeted identification of peptides difficult. By using piglet proximal jejunum extracts mounted in the UC, it was possible to take advantage of the innate proteolytically active brush border present in the tissue and detect the formation of several new peptides in the apical compartment. Only a small percentage of a peptide (from 0.6% to 3.35%) was able to cross the epithelial barrier,[57] most of them relating to hydrophobic, aromatic, and acidic residues. The results challenge the common viewpoint relating to the preferred absorption of amino acids, dipeptides, and tripeptides and support the increasing evidence of intact peptide absorption in humans. The permeability of dietary carotenoids was compared between those of mouse intestinal tissue and Caco-2 monolayers. The use of murine mucosal tissue demonstrated a nearly 2-fold uptake for all-*trans*-β-carotene from orange-fleshed sweet potato as compared to the Caco-2 cells. This murine data compares well with published human *in vivo* data placing the tissue model as a more realistic approach.[58]

Another system often used *ex vivo* is the everted small intestinal sac method, which consists of a small piece of intestine removed from anesthetized rats, flushed with buffer, and everted over a tube with the serosal part inside the sac and the mucosal side facing the buffer solution. Both ends are tied and filled with oxygenated buffer and accumulation of the sample in the inner compartment is measured. In a study which compared the transport of egg white digests, a higher permeability was measured in the everted rat sacs than in the Caco-2 cell monolayer, with lower brush border enzyme degradation occurring in the cell model.[59] However, there were some similarities; namely, four peptides with a molecular size of 8−9 amino acid residues were identified in the transported fraction in both models. The fact that both models permitted the transport of these rather long peptides increases the likelihood that these peptides are absorbed *in vivo*, although no *in vivo* data is currently available. Noneverted version of small intestinal sac is used as well, with permeability of passively transported molecules similar to that in everted sacs and permeability of actively transported molecules being lower.[60] Apparent permeability of 11 marketed drugs and 13 bioactive natural compounds in noneverted small intestinal sacs demonstrated strong correlation with reported in the literature data on human fraction absorbed. Overall, both of these methods increase throughput in absorption studies, require fewer animals, and have a lower amount of studied compound

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compared to the animal studies, being more feasible at early stages of drug discovery or food product development.

5. THE NEED TO STANDARDIZE *IN VITRO* MODELS

The absorption models based on Caco-2 alone or in combination with other intestinal cells are the most popular option in nutrition. However, the comparability of the assays between laboratories remains unknown due to the large variety of food compounds and conditions assayed. It is generally recognized that the cell source and the culture procedure can have an impact on the permeability as well as on the monolayer integrity. Thus, the heterogeneity in transporter activity of several Caco-2 clones was ascribed to differences in transporter expression, as shown for PepT1 and MDR1, and this was determined by the culture conditions.[27] Moreover the biocompatibility of complex samples such as food digesta with intestinal cell models needs particular attention.[61] This has led this group of researchers to work on the standardization of an *in vitro* model to be applied to such substrates. First, it is necessary to have a shared procedure for the manipulation of the intestinal cell lines as well as for the preparation of the cell monolayers ready for absorption experiments. For example, the source of culture media and supplements, specifically fetal bovine serum, are critical, as are the type of plastic material used and the method(s) used to determine the permeability of the cellular monolayer, among others. The source of the cell lines and their maintenance can also introduce important variability. To check this, seven groups working within the INFOGEST network made their cell lines available to determine the permeability of the generated monolayers by both automated impedance-based and manual TEER measurement (experimental details in Supporting Information "INFOGEST inter-lab TEER determination of Caco-2 and HT29-MTX monolayers") at IPLA-CSIC, Spain. A total of 11 clones, seven Caco-2 clones and four HT29-MTX, were analyzed. The results showed a strong effect of cell clone on TEER values, regardless of the equipment used for the measurement of this parameter (Figure 3). Values between Caco-2 clones could differ up to 50 times with the automated impedance measurement or 15 times with the manual ohmmeter. The differences in HT29-MTX were lower. Therefore, the origin and subsequent management of the Caco-2 and HT29-MTX clones are a source of variation and an important consideration in the proposal of a standardized absorption procedure. It is therefore critical to suggest a basal range of values that will indicate and validate the permeability state of the monolayer before undertaking any absorption experiment.

Nutrient/contaminant bioavailability is a complex multi-organ process involving several cell types and regulatory mechanisms. Several models of different physiological relevance are used to screen and study intestinal absorption, where the impact of the nature of the food compound is crucial. Current cell-based models are likely to generate results that may not be fully predictive of true bioavailability in humans. Thus, they generally do not account for the strong intersegmental variability, the variability within the population (e.g., children, elderly people, pharmacological therapies, pathologies, sex, hormonal fluctuations, etc.), as well as the interindividual variability. Animal models can also be problematic, as metabolic conversion and absorption processes are often different. However, *in vitro* models are a good starting point to provide a basis for mechanistic data on absorption and can minimize the use of animal experiments. A joint effort to agree and standardize robust protocols of intestinal transport studies of food compounds is of paramount importance to value the significance of human intestinal cell-based assays and allow the comparison of results from different laboratories. However, values of absorption *in vivo* will always be necessary to further improve *in vitro* methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c05479.

INFOGEST inter-lab TEER determination of Caco-2 and HT29-MTX monolayers (PDF)

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Notes

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