

# An Inter-laboratory Study to Evaluate the Effects of Medium Composition on the Differentiation and Barrier Function of Caco-2 Cell Lines

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**Summary** — Differentiated human intestinal Caco-2 cells are frequently used in toxicology and pharmacology as *in vitro* models for studies on intestinal barrier functions. Since several discrepancies exist among the different lines and clones of Caco-2 cells, comparison of the results obtained and optimisation of models for use for regulatory purposes are particularly difficult, especially with respect to culture conditions and morphological and biochemical parameters. An inter-laboratory study has been performed on the parental cell line and on three clonal Caco-2 cell lines, with the aim of standardising the culture conditions and identifying the best cell line with respect to parameters relevant to barrier integrity, namely, trans-epithelial electrical resistance (TEER) and mannitol passage, and of epithelial differentiation (alkaline phosphatase activity). Comparison of the cell lines maintained in traditional serum-supplemented culture medium or in defined medium, containing insulin, transferrin, selenium and lipids, showed that parameter performance was better and more reproducible with the traditional medium. The maintenance of the cell lines for 15 days in culture was found to be sufficient for the development of barrier properties, but not for full epithelial differentiation. Caco-2/TC7 cells performed better than the other three cell lines, both in terms of reproducibility and performance, exhibiting low TEER and mannitol passage, and high alkaline phosphatase activity.

**Key words:** barrier integrity, Caco-2, defined medium, epithelial differentiation, permeability, protocol standardisation.

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## Introduction

The intestinal barrier plays an important role in the protection of the whole organism against toxic substances. However, it also represents one of the major sites of exposure to potential toxicants, due to its wide extension and its physiological role in the transfer of nutrients from the lumen to the blood, and in the control of water and electrolyte movement.

In recent years, increased attention has been paid to *in vitro* models in toxicological investigations, for both ethical and scientific reasons. In the European Union, *in vitro* methods will play a major role in future legislation on chemicals (1) and also in relation to the Seventh Amendment to the *Cosmetic Directive* (2). Both of these policies call for the broad replacement, reduction and refinement of

animal experiments on a short-term basis. In some cases, the animal test can be replaced by a single alternative (for example, skin corrosion and phototoxicity). In other cases, such as systemic, repeated-dose and chronic toxicity studies, integrated testing strategies will be required that include, among others, *in vitro* models which are able to predict gut absorption and bioavailability, and thus the likelihood of systemic exposure. Therefore, a widespread interest has been shown by stakeholders such as the pharmaceutical, agro-zootechnical, food and cosmetic industries, in the use of new cell-culture systems that mimic the intestinal mucosa for investigating drug, nutrient and xenobiotic absorption and possible toxic effects.

Since primary cell cultures and cell lines from normal intestinal tissue have not, so far, been suc-

cessfully maintained in culture in a differentiated state, colon carcinoma cell lines, capable of expressing the differentiated functions of the mature absorptive intestine, have been extensively used in the field of pharmacotoxicology. The most widely used of these is the Caco-2 cell line, established by Fogh and co-workers in 1977 from a human colon adenocarcinoma, and originally used for the screening of cytotoxic effects of anti-tumour drugs and for the study of drug resistance mechanisms (3). Only later was it demonstrated that Caco-2 cells, maintained in long-term culture, undergo both morphological and functional differentiation (4–7). After confluence, the cells start to polarise, tight junctions and microvilli develop on the apical (Ap) side, and several enzymatic activities, typical of the small intestinal brush border, begin to be expressed on the Ap membrane, reaching maximum levels after 15 to 21 days from confluence (4). Specific intestinal carriers for sugars, amino acids, oligopeptides, vitamins, bile acids, micronutrients and nucleosides, have been identified and functionally characterised in Caco-2 cells (8), but with levels of expression that do not always correspond to those in the small intestine *in vivo*, partly because of the influence of several cell-related and culture-related factors (9). Moreover, these cells have been shown to exhibit numerous metabolic activities, such as those of phase I metabolic enzymes (mainly cytochrome P450 isoforms), phase II enzymes (in particular, glutathione S-transferases, an important family of detoxification enzymes that catalyse the conjugation of electrophilic substances), and of phase III transport systems (which support the active uptake into cells or promote the efflux of xenobiotics out of cells), as well as other related enzymatic activities (10–17).

Differentiated Caco-2 cells exhibit a better morphological and functional enterocytic differentiation than do other colon carcinoma cell-lines (5, 18), although the co-existence of enterocytic and colonocytic characteristics in this line is fully recognised (19, 20). The Caco-2 cell line is, at present, considered to be the best *in vitro* model of the small intestine, despite some heterogeneity in its characteristics and some limitations arising from its tumoural origin.

In toxicology, the Caco-2 cell line is used to study the effects and metabolism of natural and synthetic compounds at the intestinal level, or to investigate their transport across the epithelial barrier (21). In addition, nutrient uptake and transport studies can be performed with these cells, cultured on permeable filters (22). In pharmacology, Caco-2 cells are used to investigate the mechanisms of drug transport and biotransformation in the development of new active principles able to efficiently cross the intestinal barrier, while retaining their therapeutic properties (23).

Over the years, the original Caco-2 cell line, Caco-2/parental, has been propagated in numerous labo-

ratories around the world, giving rise to various cell lines that exhibit differences in growth characteristics and differentiation. In addition, to reduce the heterogeneity of the Caco-2/parental cell line and to improve the performance and the stability of this cellular model, some clonal cell lines have been obtained from the Caco-2/parental, and are now frequently used as an alternative to the original cell line. However, from the data available in the literature, it is evident that several discrepancies exist among the different lines and clones of Caco-2 cells as far as culture conditions and morphological and biochemical parameters are concerned, thus making difficult comparisons of the results obtained with them (9). It is therefore particularly important to standardise the Caco-2 cell model for the wide range of applications for which it is suitable, and to set up protocols for specific testing that can be further validated.

For this purpose, an international study financed by the European Centre for the Validation of Alternative Methods (ECVAM), has been carried out, with the aim of: 1) defining the characteristics of the parental Caco-2 cell line and some clonal lines, and determining minimal culture requirements for their reliable use for different purposes; 2) identifying the best cell lines and related parameters for each of the commonly-used endpoints, such as differentiation, permeability/absorption, metabolism/biotransformation, toxicity; 3) elaborating specific standard operative procedures (SOP) for each of the tests measuring relevant endpoints; and 4) establishing the relative importance of the tumoural origin of these cells with respect to their capability to differentiate (24).

This paper reports part of the results obtained in the study, and in particular, those related to the characterisation of various Caco-2 cell lines, (Caco-2/parental and the clonal lines Caco-2/TC7, Caco-2/15 and Caco-2/AQ) with respect to epithelial barrier integrity and cell differentiation, two of the most relevant endpoints for an intestinal *in vitro* model. In fact, permeability of the intestinal mucosa is a crucial characteristic of the intestine, as it determines the selectivity of the mucosa to the passage of orally-ingested molecules into the blood and lymph circulation. Such selectivity depends on properties of the epithelial cell membrane, as well as those of the tight junction complexes, the latter being influenced by physiological and toxic stimuli (25). The parameters that have been most frequently used to assess the permeability of intestinal epithelial cell monolayers are measurement either of trans-epithelial electrical resistance (TEER) or of the paracellular passage of extracellular marker molecules (for example, mannitol, inulin, phenol red, fluorescein, and polyethylene glycol) across the cell monolayer. Although the measures of TEER and of the trans-epithelial passage of marker molecules are both indicators of the integrity of the tight

junctions and of the cell monolayer, they determine different entities. TEER reflects the ionic conductance of the paracellular pathway in the epithelial monolayer, while the flux of non-electrolyte tracers (expressed as apparent permeability, Papp) indicates the paracellular water flow, as well as the pore size of the tight junction (26).

In the small intestine, the differentiation of the enterocytic cells during migration from the crypts to the tip of the villi results in an increased expression of some enzymes, such as disaccharidases, dipeptidases and alkaline phosphatase. Increased expression following differentiation has also been demonstrated in Caco-2 cells *in vitro* for some brush-border enzymes (22), including alkaline phosphatase (27), which has biochemical characteristics similar to those of the intestinal isoenzyme (5).

In this inter-laboratory study, TEER and mannitol passage (MAN) were selected as markers of epithelial barrier integrity, and alkaline phosphatase (AP) as a differentiation marker, and the respective testing protocols have been optimised.

The study was performed in five different laboratories experienced in working with Caco-2 cells, with shared common competences, but also with specific expertise: Istituto Superiore di Sanità, Rome, Italy (ISS); Inserm U505/UPMC, Institut Biomédical des Cordeliers, Paris, France (INSERM); Department of Pathophysiology, Medical University of Vienna, Vienna, Austria (DPMUW); GlaxoSmithKline, Verona, Italy (GSK); Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, Rome, Italy (INRAN); and Sanofi-Synthelabo Research, Montpellier, France (SSR).

## Materials and Methods

### Materials for cell culture

Dulbecco's Modified Eagle's Medium without Napyruvate (DMEM), fetal calf serum (FCS), glutamine, non-essential amino acids, penicillin/streptomycin, insulin/transferrin/selenium (ITS) supplement, were obtained from Euroclone, Milan, Italy, or Gibco BRL, Gaithersburg, MD, USA. N-(2-hydroxy-ethyl)piperazine-N'-2-etane-sulphonic acid (HEPES) was obtained from Sigma Chemical Co., St Louis, MO, USA, or Gibco BRL, and fatty acid-free bovine serum albumin (BSA), cholesterol, oleic and palmitic acid from Sigma Chemical.

### Other materials

p-Nitrophenol (pNP) and p-nitrophenyl phosphate (pNPP) were purchased from Sigma Chemical, and D-1-[<sup>3</sup>H(N)] mannitol (specific activity 555GBq-

1.11TBq/mmol) from NEN (Perkin Elmer Life and Analytical Sciences, Milan, Italy). All other chemicals were of analytical grade.

### Cell lines

Four cell lines were used in this study: the Caco-2/parental cell line (Caco-2/ATCC cat no. HTB-37) and three clones: Caco-2/TC7 (provided by INSERM), a clone derived from Caco-2/parental cells at passage 180, expressing high taurocholic acid transport and Cyp3A activity (28); Caco-2/15 (provided by DPMUW), a slow-growing sub-clone obtained by dilution plating from Caco-2/parental, exhibiting high sucrase-isomaltase activity (29) and considered to be a relatively differentiated tumour cell line; and Caco-2/AQ (provided by DPMUW), sub-cloned from Caco-2/15 at passage 100, highly proliferative, with high TEER and alkaline phosphatase values (30) and considered to be a rather undifferentiated tumoural cell line.

### Growth conditions

To provide for good data reproducibility, the Caco-2 cells were used within the same range of passage numbers in the different laboratories: Caco-2/parental cells were used between passages 14 and 30, Caco-2/AQ and Caco-2/15 between passages 4 and 20, and Caco-2/TC7 between passages 60 and 75.

The cells were sub-cultured once a week, at about 80% confluence, and the medium was changed three times a week. Since growth rates varied in the different laboratories, possibly due to the use of different batches of FCS, cell-seeding densities were selected for each cell line so that they could be sub-cultured once a week. The following ranges were used:  $7-15 \times 10^3$  cells/cm<sup>2</sup> (Caco-2/parental),  $2-4 \times 10^3$  cells/cm<sup>2</sup> (Caco-2/TC7),  $2.5-5 \times 10^3$  cells/cm<sup>2</sup> (Caco-2/AQ), and  $2.5-5 \times 10^3$  cells/cm<sup>2</sup> (Caco-2/15). The cells were seeded at higher density (about  $2-4 \times 10^5$  cells/cm<sup>2</sup>) on transparent polyethylene terephthalate (PET) filter inserts than on conventional plastic supports, in order to achieve cell confluence after 2-3 days in every laboratory.

### Media

The parental cell line and the three clones were routinely grown in basal DMEM with high glucose (4500mg/l), 4mM glutamine, 1% non-essential amino acids, 10mM HEPES, 100IU/ml penicillin and 100µg/ml streptomycin, supplemented with heat-inactivated FCS (20% for the first two days after plating, and then 10%), and were kept at 37°C in 5% CO<sub>2</sub>.

For TEER, MAN and AP measurements, the cells were seeded on transparent PET inserts in

12-well plates (0.9cm<sup>2</sup> growth area, 1µm pore size; BD Falcon tissue culture inserts, Becton Dickinson Labware, Franklin Lakes, NJ, USA). For the first 3 days after seeding, the cells were maintained in DMEM, containing 10% FCS, in both apical (Ap) and basolateral (Bl) compartments (DMEM/FCS-sym), to allow for uniform growth conditions. At confluence on day 4, the medium was changed, either to the usual FCS-containing medium conditions (DMEM/FCS-sym) or to a defined medium consisting of DMEM without FCS in the Ap compartment, and DMEM supplemented with ITS, 1% BSA and with 1% lipid mixture (L: cholesterol 0.04mM, oleic acid 0.4mM and palmitic acid 0.2mM) in the Bl compartment (DMEM/ITS/L-asyM).

### DMEM/ITS/L preparation

Stock solutions of oleic acid (100mM), palmitic acid (100mM) and cholesterol (50mM) were made in chloroform/methanol (4/1) in screw-capped glass tubes (with a Teflon joint), and aliquots were maintained in the dark at -20°C. To supplement 100ml of medium, 400µl of oleic acid, 200µl of palmitic acid and 80µl of cholesterol from the stock solutions were mixed in a screw-capped tube, and the solvent was eliminated under N<sub>2</sub> flux at 45°C. The lipid film was resuspended in 10ml of 1% BSA in DMEM. 10ml of this solution was emulsified by ultrasonication for 10 minutes, and then gradually added to 90ml of the 1% BSA/DMEM solution, and mixed by stirring for 2 to 3 hours at 37°C, until a clear solution was obtained. The medium was then filter sterilised through a 0.22µm membrane filter (31).

### TEER

TEER measurements were performed on day 15, 18 and 21 of culture, by using two different measuring devices.

*Chamber.* TEER values were recorded via a chamber with concentric electrodes (manufactured by DPMUW) or via an EVOM epithelial voltohmmeter with electrode STX2 (World Precision Instruments, Berlin, Germany). Three separate measurements were performed in the culture medium at each of two temperatures, 25–30°C and 37°C.

*Chop-stick electrodes.* TEER values were recorded by using the Millicel-ERS apparatus (Millipore Co., Bedford, MA, USA). Three separate measurements were performed in the culture medium for each insert, at 37°C.

In all cases, TEER values were expressed as Ohm (Ω) × cm<sup>2</sup> according to the following equation:

$$\text{TEER} = [\Omega \text{ cell monolayer} - \Omega \text{ filter (cell-free)}] \times \text{filter area.}$$

All the participants observed that TEER values increased after medium renewal. Hence, it was agreed to make the measurements one day after the renewal procedure.

### Mannitol passage (MAN)

D-1-[<sup>3</sup>H(N)] mannitol, a hydrophilic extra-cellular space marker, was used to evaluate the passive transport across the epithelial cell monolayer. Measurements were performed on day 15, 18 and 21 of culture. For the assay, the growth medium in the Ap compartment was discarded and substituted with 0.5ml DMEM, without serum, supplemented with 25µM D-1-[<sup>3</sup>H(N)] mannitol (specific activity 555GBq–1.11TBq/mmol). The Bl compartment received 1ml DMEM without serum. After 1 hour, the Bl medium was collected, and the radioactivity was measured in a liquid scintillation counter. Mannitol passage was expressed as the apparent permeability coefficient (Papp), according to the following equation:

$$\text{Papp (cm/s)} = \frac{V}{A \times dt} \times \frac{dC}{C_0}$$

Where V = volume of the Bl acceptor chamber (cm<sup>3</sup>); A = filter area (cm<sup>2</sup>); t = time (s); dC = mannitol concentration in the Bl chamber at time t; and C<sub>0</sub> = mannitol concentration in the Ap chamber at time zero.

### Alkaline phosphatase (AP) activity

AP activity was determined after 15, 18 and 21 days of culture. Cell monolayers were washed twice with cold phosphate-buffered saline (PBS, pH 7.4) and were then dissolved with 0.5% Triton X-100 in 10mM TRIS-HCl and 150mM NaCl (pH 8.0). Then 150µl of each sample was added to 450µl of p-NPP and incubated at 37°C. The reaction time was adjusted to fall within the linear range of enzymatic activity of the sample. The reaction was stopped by the addition of 150µl 0.5M NaOH, and the absorbance was measured at 405nm excitation wavelength/490nm emission wavelength.

The specific enzymatic activity was related to a standard curve of the reaction product p-NP and was expressed as mU/mg of protein (1mU = 1nmol of pNP/min, at 37°C).

### Total protein content

The total protein content of the cell extract samples used for the alkaline phosphatase assay was measured according to one of three methods: the Lowry

method (32); the Bradford colourimetric method (33); or the BCA kit (Pierce, Rockford, IL, USA).

### Statistical analysis

For each of the three parameters under investigation, either three (MAN) or six (AP and TEER) laboratories generated data by using four cell types, two culture conditions and three different days of culture. Each parameter was measured in triplicate, and in three identical and independent experiments. In view of the number of variables (laboratory, cell type, medium, experiment, and days in culture), a hierarchical analysis, focusing stepwise on several aspects, was conducted, in which the data were logarithmically transformed throughout, approximating normality and variance homogeneity of data. All calculations were performed with either S-Plus 6.1 (© Insightful, Seattle, WA, USA) or GraphPad Prism 4 (San Diego, CA, USA).

#### *Trend analysis*

The effects of the time in culture on each parameter were analysed. A repeated measurement ANOVA was performed for each laboratory-parameter combination, at three time-points, namely 15, 18 and 21 days after seeding. If an overall significant trend over time was found, the same technique was employed to explore the data in detail with respect to cell type and serum condition. As only a clear and strong effect over time would justify a more cost-intensive and time-intensive prolongation of the culture time, the level of significance was set at 1%; i.e. only  $p$ -values smaller than 1% were considered to be an indication of a trend.

#### *Within-laboratory reproducibility*

A three-way or, where only one medium was tested, two-way ANOVA without interactions with the independent variables “experiment”, “cell” and “medium”, was calculated for each laboratory. First, the relative mean square error of the variable “experiment”, which is expected to be small, was used to describe its contribution to the overall variation in the ANOVA. This variable was considered to be reproducible, if it had a  $p$ -value greater than 1%. Otherwise, the data were analysed in detail with a one-way ANOVA for every cell type-medium combination, in order to assess their reproducibility, where, again, a  $p$ -value larger than 1% was considered to be reproducible. Additionally, for every combination, the coefficient of variation (CV) of the experiment means was calculated, permitting a

more detailed insight into the within-laboratory reproducibility.

#### *Performance analysis*

For each parameter (TEER, MAN, AP), the three factors “medium”, “cell” and “laboratory”, were analysed by using a three-way ANOVA model without interactions, mainly considering the differences between media and between cell types. In the case of a significant medium effect, again to a significance level of 1%, a two-way ANOVA was calculated separately for the medium conditions and the different cell types. The cell types were subsequently compared pair-wise with the Bonferroni *post-hoc* test. When the results from the medium conditions were not significant, the model was reduced, and the two-way ANOVA was performed directly, i.e. without separating the data set according to the medium conditions.

## Results

### Growth conditions

The protocol for cell maintenance reported in Materials and Methods was adopted by the six laboratories after extensive discussions, and was shown to be suitable for the Caco-2/parental cell line and for the three clonal cell lines. Due to the different batches of culture materials used by the participants, it was not possible to adopt the same seeding density in plastic flasks in each laboratory, but each participant established the in-house optimal conditions needed for the cells to be sub-cultured once a week. On PET filter inserts the four cell lines, seeded at higher densities, reached confluence in 2 to 3 days in all six laboratories.

### TEER measurements

#### *Trend analysis*

TEER values were determined for each of the four cell types cultured either in DMEM/FCS-sym (six laboratories) or in DMEM/ITS/L-asym (five laboratories). The measurements were carried out on days 15, 18 and 21 after seeding, in order to verify, by trend analysis, whether this parameter changed with the number of days in culture. The resulting  $p$ -values for the repeated measures ANOVA, shown in Table 1, revealed a significant effect of time on TEER in only one laboratory (namely, ISS).

**Table 1: Trend analysis for all laboratory-parameter combinations for days in culture**

Laboratory	TEER	MAN	AP
DPMUW	0.5838	–	0.3149
GSK <sup>a</sup>	0.0629	–	0.8326
INRAN	0.1173	0.0878	0.1130
INSERM <sup>b</sup>	0.1334	–	< 0.0001 <sup>d</sup>
ISS	< 0.0001 <sup>d</sup>	0.0031 <sup>d, e</sup>	0.0051 <sup>d, e</sup>
SSR <sup>c</sup>	0.2297	0.0092 <sup>d, e</sup>	< 0.0001 <sup>d</sup>

Values are *p*-values for a repeated measurement ANOVA, measured at days 15, 18 and 21 after seeding.

<sup>a</sup>DMEM/ITS/L-*asym* only; <sup>b</sup>mainly two experiments only; <sup>c</sup>DMEM/FCS-*sym* only; <sup>d</sup>significant ( $p < 0.01$ ); <sup>e</sup>non-significant effects in the detailed analysis.

TEER = trans-epithelial electrical resistance, MAN = mannitol passage, AP = alkaline phosphatase activity.

The detailed analysis (i.e. repeated ANOVA for each combination of medium and cell line) of the data from ISS showed that the combination of Caco-2/parental with DMEM/ITS/L-*asym* caused this significant effect, due to a lower response at day 15. Overall, since no marked and consistent trend was detected, the TEER data on the different days were combined and are presented in Figure 1, according to laboratory and medium, and showing each cell line in a separate section.

#### Within-laboratory reproducibility

Focusing on the variation of TEER values within the laboratories, good within-laboratory reproducibility was observed for GSK, ISS and SSR, i.e. non-significant experimental effects were found (*p*-values above 0.08 and relative mean squares around 1%; Table 2).

**Table 2: *p*-value and relative mean square error for the variable “experiment”, for each laboratory and parameter**

Parameter	Laboratory error (%)	<i>p</i> -value	Relative mean square
TEER	DPMUW	< 0.001 <sup>b</sup>	2.1
	GSK <sup>a</sup>	0.298	1.1
	INRAN	< 0.001 <sup>b</sup>	2.4
	INSERM	< 0.001 <sup>b</sup>	5.8
	ISS	0.087	1.0
	SSR <sup>a</sup>	0.124	0.9
MAN	INRAN	0.042	0.8
	ISS	< 0.001 <sup>b</sup>	12.0
	SSR <sup>a</sup>	0.396	4.6
AP	DPMUW	< 0.001 <sup>b</sup>	32.1
	GSK <sup>a</sup>	0.004 <sup>b</sup>	2.7
	INRAN	0.002 <sup>b</sup>	1.0
	INSERM	0.108	0.9
	ISS	< 0.001 <sup>b</sup>	7.9
	SSR <sup>a</sup>	0.570	1.6

Values are obtained from an additive three-way ANOVA.

<sup>a</sup>only one kind of medium, thus a 2-way ANOVA was applied; <sup>b</sup>significant ( $p < 0.01$ ).

TEER = trans-epithelial electrical resistance, MAN = mannitol passage, AP = alkaline phosphatase activity.

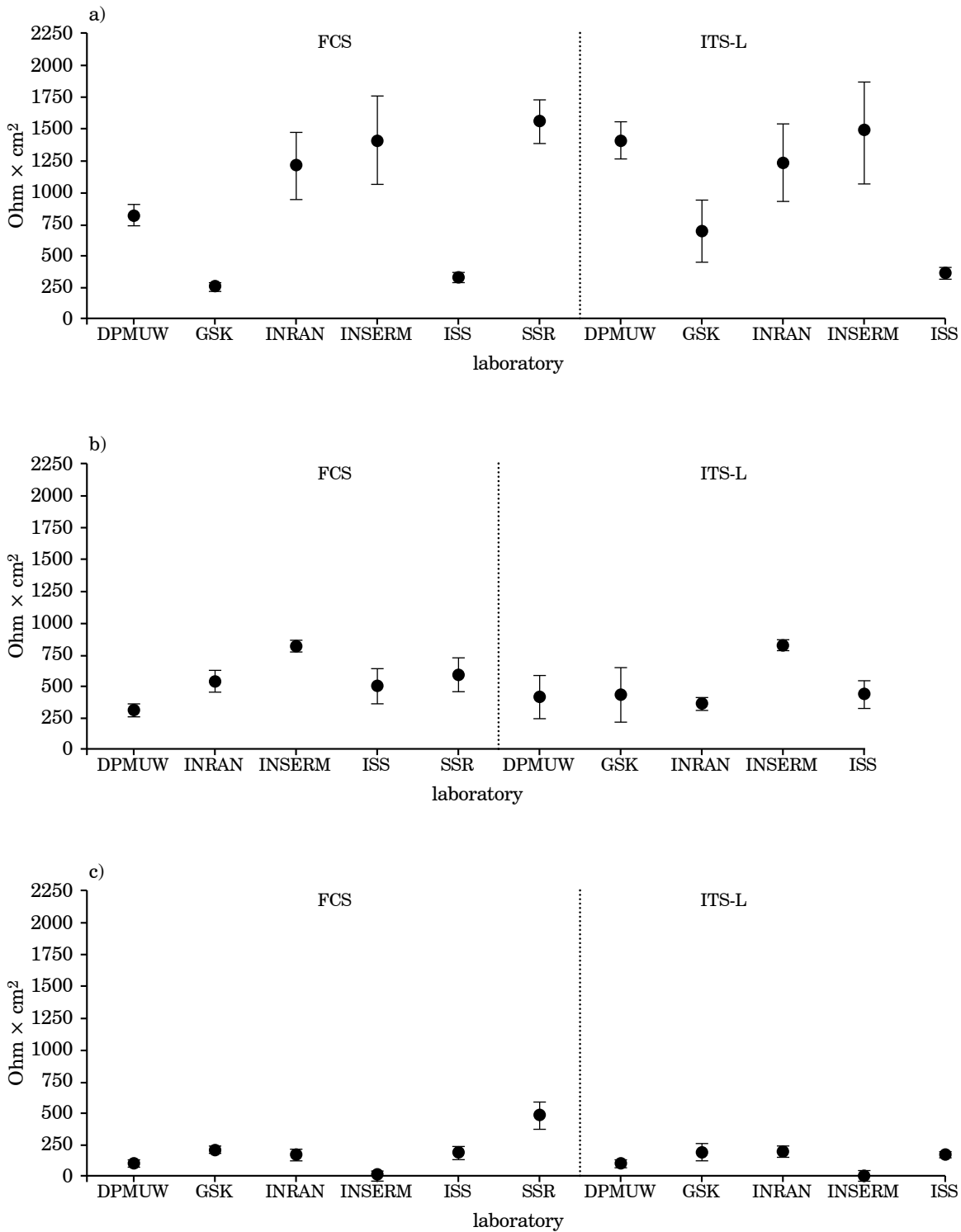
Detailed analyses were carried out for the laboratories that showed reduced reproducibility. In DPMUW, this revealed CVs below 30% for all combinations, except for Caco-2/parental in DMEM/ITS/L-*asym* (CV 50%) (Table 3). At INRAN, experiments performed in DMEM/FCS-*sym* were more reproducible for Caco-2/parental and Caco-2/15 (CVs below 6%) than for Caco-2/AQ (CV = 24.6%) and Caco-2/TC7 (CV = 16.4%). With DMEM/ITS/L-*asym*, the results obtained with Caco-2/parental and Caco-2/AQ were reproducible, while those obtained with Caco-

**Table 3: Coefficients of variation between experiments for trans-epithelial electrical resistance (TEER), according to medium, laboratory and cell line**

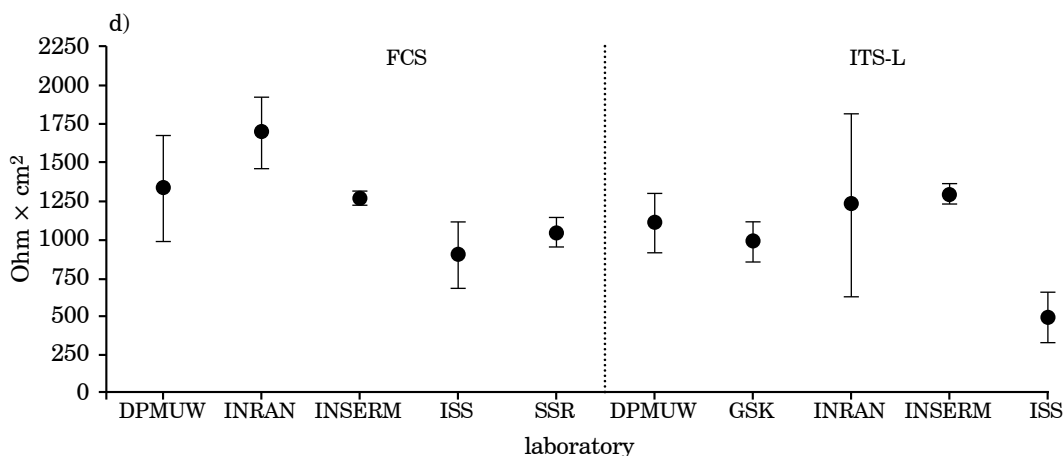
Laboratory	FCS						ITS-L				
	DPMUW	GSK <sup>a</sup>	INRAN	INSERM	ISS	SSR	DPMUW	GSK	INRAN	INSERM	ISS
<b>Cell type</b>											
Caco-2/AQ	7.0 <sup>c</sup>	4.1	24.6 <sup>c</sup>	28.6 <sup>c</sup>	6.3	1.6	9.9 <sup>c</sup>	29.6	9.3	32.5 <sup>c</sup>	4.4
Caco-2/parental	14.9 <sup>c</sup>	—	5.1	0.7 <sup>b</sup>	10.0	7.2	50.0 <sup>c</sup>	57.0	8.3	0.6 <sup>b</sup>	6.7
Caco-2/TC7	24.0 <sup>c</sup>	3.9	16.4 <sup>c</sup>	14.1 <sup>c</sup>	22.2 <sup>c</sup>	1.7	17.3 <sup>c</sup>	33.1	19.0 <sup>c</sup>	5.2 <sup>c</sup>	12.8
Caco-2/15	28.0 <sup>c</sup>	—	5.7	1.1 <sup>b</sup>	8.1	1.9	12.2 <sup>c</sup>	10.1	56.1 <sup>c</sup>	0.6 <sup>b</sup>	17.5

FCS = DMEM/FCS-*sym* medium, ITS-L = DMEM/ITS/L-*asym* medium; <sup>a</sup>only day 15; <sup>b</sup>two experiments only; <sup>c</sup> $p < 0.01$  in detailed ANOVA.

**Figure 1: Trans-epithelial electrical resistance (TEER) values of the four Caco-2 cell lines maintained in two different media, measured in the different laboratories**



Each section shows the pooled TEER values, measured at days 15, 18 and 21 from seeding, of a single cell line maintained in DMEM/FCS-sym medium (FCS) in six laboratories and in DMEM/ITS-L-asy (ITS-L) in five laboratories. a) Caco-2/AQ; b) Caco-2/parental; c) Caco-2/TC7. Each value represents the mean  $\pm$  SD of three pooled TEER values from each of three independent experiments. TEER values are expressed as  $\text{Ohm} \times \text{cm}^2$ .

**Figure 1: continued**

Each section shows the pooled TEER values, measured at days 15, 18 and 21 from seeding, of a single cell line maintained in DMEM/FCS-sym medium (FCS) in six laboratories and in DMEM/ITS-L-asm (ITS-L) in five laboratories. d) Caco-2/15. Each value represents the mean  $\pm$  SD of three pooled TEER values from each of three independent experiments. TEER values are expressed as Ohm x cm<sup>2</sup>.

2/TC7 and Caco-2/15 were not (CVs of 19.0% and of 56.1%, respectively). At INSERM, Caco-2/AQ (CV around 30%) and Caco-2/TC7 (CV around 13%) revealed significant differences among experiments for both media, whereas Caco-2/parental and Caco-2/15, only tested in two experiments, showed non-significant differences. As the relative mean square error for the variable “experiment” was lower than 2.5% for all the laboratories, except for INSERM (5.8%; Table 2), the data from the three experiments were pooled in subsequent analyses.

#### Performance analysis

In the final analysis, the medium conditions did not exert a significant effect on TEER in the three-way ANOVA ( $p$ -value = 0.067). The reduced model with the *post-hoc* Bonferroni comparison showed that the lowest TEER values were obtained with Caco-2/TC7 or Caco-2/parental cells in all the laboratories. Focusing on these two cell lines, Caco-2/TC7 gave significantly lower values than Caco-2/parental cells in four out of six laboratories. In the other two laboratories (INSERM and SSR), no significant differences in TEER values were observed between these two cell lines. The other two cell lines, Caco-2/AQ and Caco-2/15, had much higher and very variable TEER values, and thus were considered to be more distant from the *in vivo* situation in the small intestinal epithelium.

#### MAN passage

##### Trend analysis

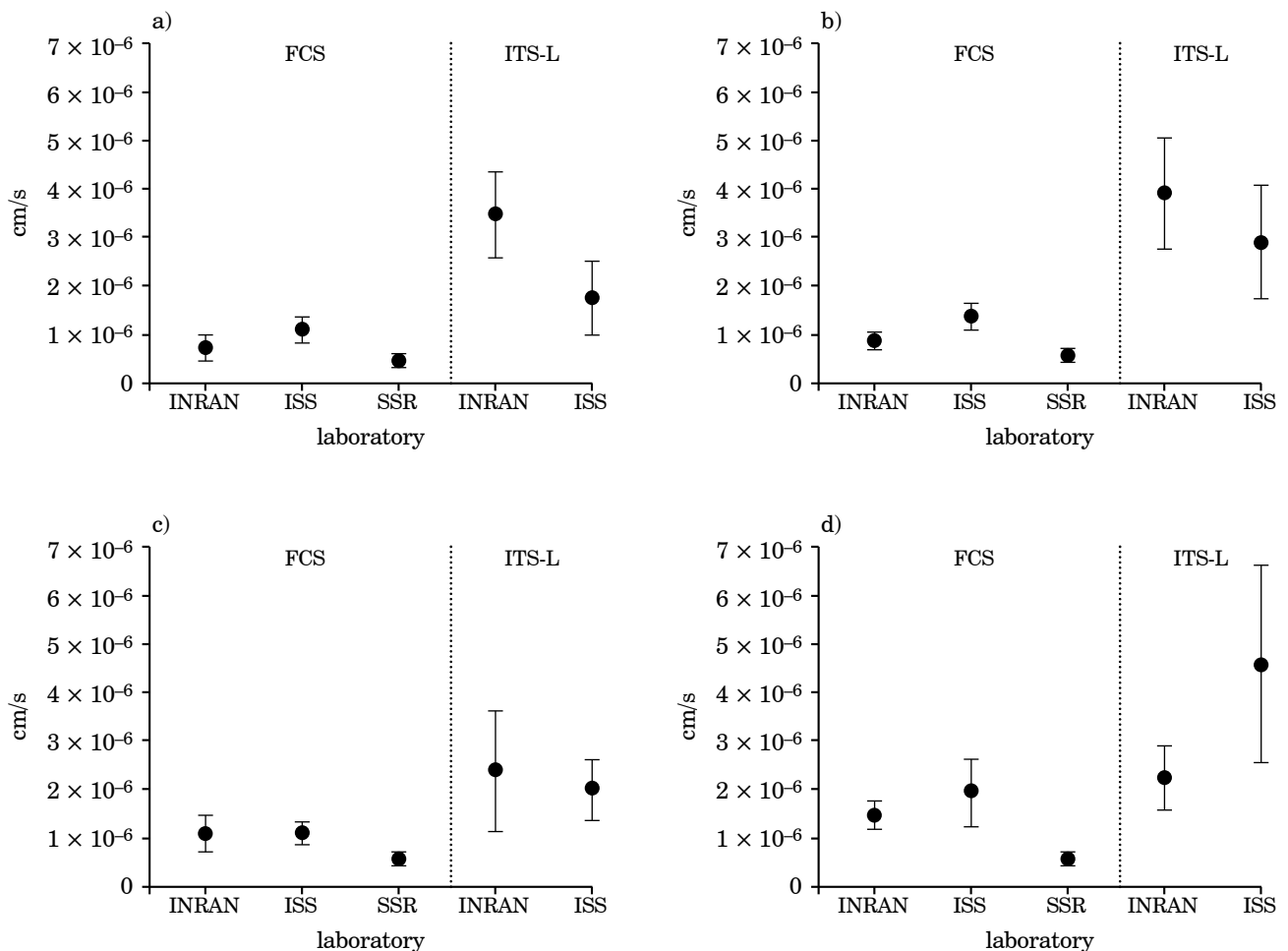
MAN passage was measured at the same time points as for TEER measurements. Due to the need for a radioactive probe in this analysis, only three laboratories (INRAN, ISS and SSR) were involved in these experiments (Figure 2). As shown in Table 1, the trend analysis with respect to the number of days in culture (15, 18 or 21 days) revealed a significant effect for ISS and SSR. At ISS, this was mainly caused by two experiments, in which the Caco-2/15 and Caco-2/parental lines showed an unusually high response at day 15, which subsequently decreased at days 18 and 21. For SSR, this significant effect was more difficult to interpret. As no clear trend was observed over this time-period for any combination of cell line and medium, in the detailed analysis for either laboratory, the data obtained on the different days were pooled and are presented in Figure 2, according to laboratory and medium, showing each cell line in a separate section.

##### Within-laboratory reproducibility

The INRAN and SSR data showed no significant experiment effects for MAN (Table 2). In DMEM/FCS-sym medium, all the cell lines had small CVs at



**Figure 2: Mannitol passage (MAN) of the four Caco-2 cell lines maintained in two different media, measured in the different laboratories**



Each section shows the pooled MAN values, measured at days 15, 18 and 21 from seeding, of a single cell line maintained in DMEM/FCS-sym medium (FCS) in three laboratories and in DMEM/ITS-L-asm (ITS-L) in two laboratories. a) Caco-2/AQ; b) Caco-2/parental; c) Caco-2/TC7; d) Caco-2/15. Each value represents the mean  $\pm$  SD of three pooled MAN values from each of three independent experiments. MAN values are expressed as the Papp (cm/s).

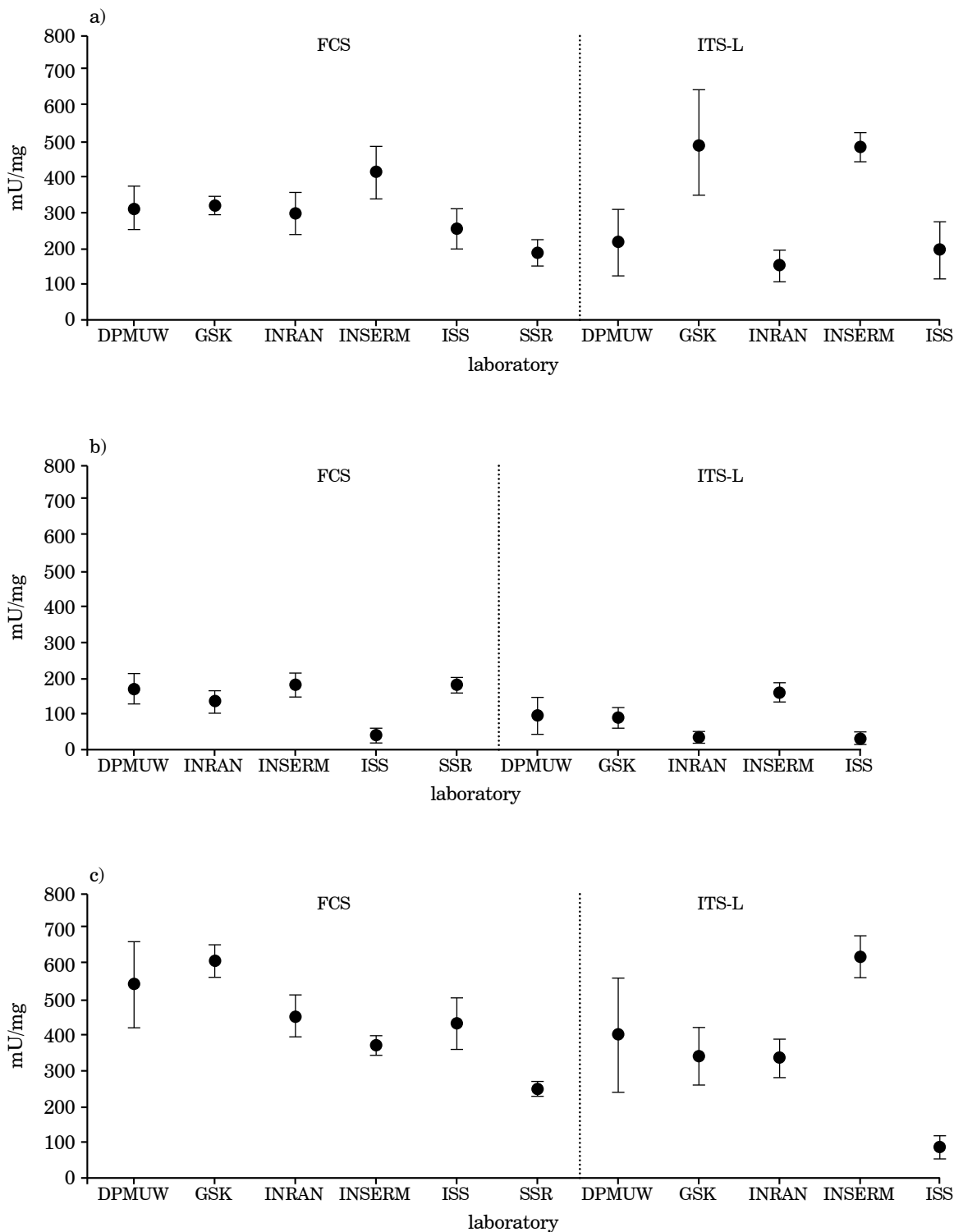
SSR (< 10%), while at INRAN, only Caco-2/parental and Caco-2/15 had CVs < 10%; the other cell lines showed much higher CVs (Table 4). Similarly, at ISS, results for Caco-2/parental and Caco-2/15 in DMEM/FCS-sym medium were more reproducible than those for Caco-2/AQ and Caco-2/TC7 (Table 4). For all the cell lines and all the laboratories, the CVs obtained in DMEM/ITS/L-asm were higher (between 22.3 and 50.2%) than those in DMEM/FCS-sym (all smaller than 25%; Table 4). Since the relative mean square errors of the variable “experiment” were below 5% for INRAN and SSR, and 12% for ISS (Table 2), the results from the three experiments were combined in subsequent analyses.

#### Performance analysis

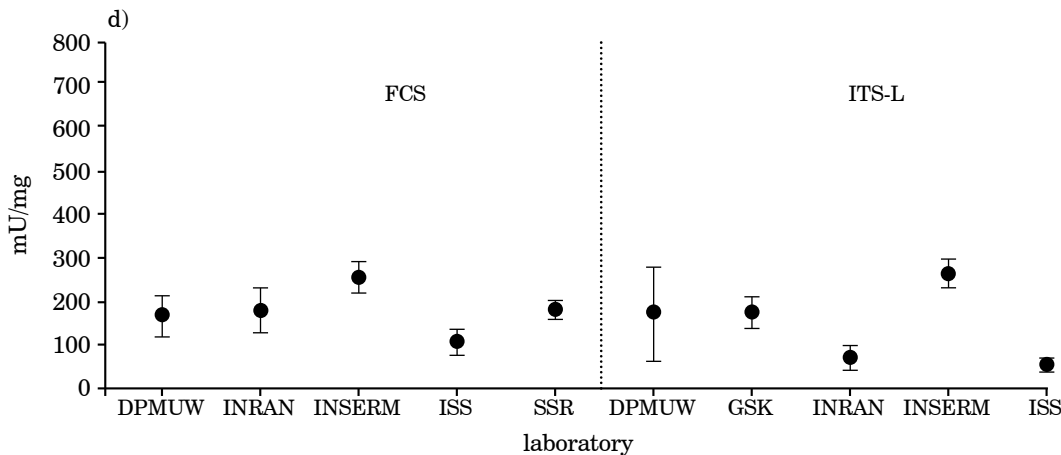
Performance analysis of the MAN parameter revealed a highly significant effect of the medium: maintenance in DMEM/FCS-sym consistently resulted in lower MAN values for all the cell lines compared to DMEM/ITS/L-asm medium (Figure 2). A detailed comparison of the cell types maintained in DMEM/FCS showed that Caco-2/AQ significantly gave the lowest MAN values in each of the three laboratories, except when compared to Caco-2/TC7 at ISS, and to Caco-2/15 at SSR.

In DMEM/ITS/L-asm medium, Caco-2/TC7 gave significantly lower MAN values compared to the other cell lines, with the exception of Caco-2/AQ at

**Figure 3: Alkaline phosphatase (AP) activity of the four Caco-2 cell lines maintained in two different media, measured in the different laboratories**



Each section reports the pooled AP values, measured at days 15, 18 and 21 from seeding, of a single cell line maintained in either DMEM/FCS-sym medium (FCS) in six laboratories or in DMEM/ITS-L-asym (ITS-L) in five laboratories. a) Caco-2/AQ; b) Caco-2/parental; c) Caco-2/TC7. Each value represents the mean  $\pm$  SD of three pooled AP values from each of three independent experiments. AP values are expressed as mU/mg of protein.

**Figure 3: continued**

Each section reports the pooled AP values, measured at days 15, 18 and 21 from seeding, of a single cell line maintained in either DMEM/FCS-sym medium (FCS) in six laboratories or in DMEM/ITS-L-asm (ITS-L) in five laboratories. d) Caco-2/15. Each value represents the mean  $\pm$  SD of three pooled AP values from each of three independent experiments. AP values are expressed as mU/mg of protein.

ISS, and Caco-2/15 at INRAN. Moreover, results obtained in DMEM/ITS/L-asm medium appeared more variable, due to lower within-laboratory reproducibility, as indicated by a median CV of all laboratory–cell type combinations of 29.3%, compared to 8.5% for the DMEM/FCS-sym medium.

### AP activity

#### Trend analysis

AP activity was measured on the same days in culture (namely days 15, 18 and 21) as for the other parameters (Figure 3). The trend analysis for time in culture resulted in significant  $p$ -values for ISS, INSERM and SSR (Table 1). However, in the detailed analysis, no significant differences were observed in the data obtained at ISS. At INSERM, the increasing enzyme activity over time was more evident in DMEM/ITS/L-asm medium ( $p$ -value < 0.0001) than in DMEM/FCS-sym medium ( $p$ -value = 0.0063), although the average relative-increases from day 15 to day 21 were 32% for DMEM/ITS/L-asm and 19% for DMEM/FCS-sym. At SSR, where only DMEM/FCS-sym was tested, a clear increase in enzyme activity over time was observed in all the cell lines, with  $p$ -values for the individual cell lines lower than 0.004. Comparing the results for days 15 and 21, on average, an increase of 26.9% in the enzyme activity was found, which was most pronounced for the Caco-2/AQ cells (50.1%) and least

pronounced for Caco-2/TC7 cells (4.5%). However, this increase may not be as relevant, because it was observed over a relatively limited range of values (i.e. 130 to 300mU/mg).

#### Within-laboratory reproducibility

The SSR and INSERM laboratories showed good reproducibility of AP data, as shown by the non-significant experimental effect in the three-way

**Table 4: Coefficients of variation between experiments for mannitol passage (MAN), according to medium, laboratory and cell line**

Medium	FCS			ITS-L	
	INRAN	ISS	SSR <sup>a</sup>	INRAN	ISS
<b>Cell type</b>					
Caco-2/AQ	35.6 <sup>b</sup>	23.0 <sup>b</sup>	9.6	27.1 <sup>b</sup>	45.2 <sup>b</sup>
Caco-2/parental	5.4	6.4	3.9	23.0 <sup>b</sup>	22.3
Caco-2/TC7	40.8 <sup>b</sup>	18.3 <sup>b</sup>	5.4	47.5 <sup>b</sup>	30.6 <sup>b</sup>
Caco-2/15	7.3	22.3	6.2	11.0	50.3

FCS = DMEM/FCS-sym medium; ITS-L = DMEM/ITS/L-asm medium; <sup>a</sup>two replicates only; <sup>b</sup> $p < 0.01$  in detailed ANOVA.

ANOVA. Detailed analyses of DPMUW and ISS data showed poor reproducibility in both these laboratories for almost all cell type–medium combinations (Table 5). At INRAN, when using DMEM/FCS-sym, the Caco-2/parental data were less reproducible, with a CV of 16.4%, while those for the other cell lines had CVs below 12%. In DMEM/ITS/L-asym, the results obtained with Caco-2/TC7 were reproducible, while those of the other cell types were not reproducible, with CVs around 30%. At GSK, poor reproducibility was observed for all the cell types cultured in DMEM/ITS/L-asym medium, with CVs around 30%. Although the relative mean square of the experimental variability was 32% at DPMUW and about 8% at ISS (below 3% in the other laboratories; Table 5), the results were combined for all experiments, despite the variance-increasing effect of the DPMUW data (Figure 3).

#### Performance analysis

Performance analysis of the AP results showed a significant effect of medium type. The comparison of the two conditions in a detailed analysis for every laboratory and cell type by ANOVA, revealed ambiguous results: in DMEM/ITS/L-asym medium compared to DMEM/FCS-sym medium, the AP values were significantly lower for nine laboratory–cell combinations, and higher for two combinations, while no differences were found for the remaining five combinations. Considering maintenance in DMEM/FCS-sym medium, the multiple comparisons revealed that Caco-2/TC7 cells gave significantly higher responses than the other cell lines in five out of six laboratories, while non-significant differences were detected when comparing them with Caco-2/AQ at INSERM. Caco-2/AQ gave the second highest response, followed by Caco-2/parental and Caco-2/15. With DMEM/ITS/L-asym medium, the results were similar: except for GSK-

Caco-2/AQ and INSERM-Caco-2/AQ, Caco-2/TC7 cells significantly gave the highest response, followed by Caco-2/AQ, Caco-2/15 and Caco-2/parental.

## Discussion

According to ECVAM, one of the criteria necessary for a method to enter the validation process is an optimised protocol and the related SOPs (34). An additional problem with respect to the validation of the human intestinal Caco-2 cell line as a model of the small intestinal epithelium, is the availability of several different cell lines, often of clonal origin, that have been obtained from the parental Caco-2 cell line and are currently used in many laboratories around the world, under different experimental conditions and for various applications (9). Therefore, the main focus of this paper was to compare the characteristics of the parental Caco-2 cell line and three clonal cell lines, in order to optimise the protocols for culture conditions, by evaluating, in detail, seeding densities, medium composition, sub-culture schedules, etc. Moreover, protocols have also been optimised for crucial endpoints for intestinal cells, such as barrier integrity, evaluated by electrical (TEER) and exclusion parameters (MAN), and a differentiation marker (AP). These endpoints have been used in a comparison of the four Caco-2 cell lines under investigation.

Concerning cell culture protocols, the partners agreed on seeding densities that would achieve 80% confluence within one week, at which time the cells would be sub-cultured. With respect to culture media, the optimum for standardisation purposes would be the use of a defined medium. For this reason, the traditional medium (DMEM/FCS-sym) was compared to a synthetic medium (DMEM/ITS/L-asym), that was shown to support morphological and functional characteristics similar to those obtained with DMEM/FCS-sym, even if the latter

**Table 5: Coefficients of variation between experiments for alkaline phosphatase activity (AP), according to medium, laboratory and cell line**

Medium	FCS						ITS-L					
	Laboratory	DPMUW	GSK <sup>a</sup>	INRAN	INSERM <sup>b</sup>	ISS	SSR	DPMUW	GSK	INRAN	INSERM <sup>b</sup>	ISS
<b>Cell type</b>												
Caco-2/AQ		13.8	8.9	10.9	22.3	28.7 <sup>c</sup>	3.9	50.8 <sup>c</sup>	37.3 <sup>c</sup>	25.9 <sup>c</sup>	2.0	46.0 <sup>c</sup>
Caco-2/parental		24.2 <sup>c</sup>	–	16.4	2.2	49.2 <sup>c</sup>	0.7	65.6 <sup>c</sup>	24.6 <sup>c</sup>	33.2 <sup>c</sup>	0.5	60.1 <sup>c</sup>
Caco-2/TC7		19.1 <sup>c</sup>	7.8	2.4	5.7	12.8	0.8	47.3 <sup>c</sup>	27.2 <sup>c</sup>	3.5	0.8	29.7 <sup>c</sup>
Caco-2/15		31.3 <sup>c</sup>	–	11.2 <sup>c</sup>	2.3	28.2 <sup>c</sup>	1.9	68.4 <sup>c</sup>	22.6 <sup>c</sup>	28.3 <sup>c</sup>	0.4	27.9 <sup>c</sup>

FCS = DMEM/FCS-sym medium; ITS-L = DMEM/ITS/L-asym medium; <sup>a</sup>only day 15; <sup>b</sup>two experiments only; <sup>c</sup>p < 0.01 in detailed ANOVA.

gave better and more reproducible results (24). The lower reproducibility of the results obtained in the synthetic medium, also reported in the present paper, may be related to the delicate procedure involved in the preparation of ITS/L, which had previously been routinely performed in only one laboratory (INSERM) and which requires further optimisation and standardisation.

In this multi-laboratory study, the experimental conditions that produced the best results according to the different parameters with respect to performance, robustness and practicability, were clearly identified. For the purpose of statistical analysis, a close-to-reality performance of the parameters was assumed as optimal in the evaluation of the different experimental designs. A low response for TEER and MAN, and a high response level for AP activity were considered as optimal for a small intestinal absorptive cell model. With respect to TEER, it has been noted that the colonic origin of the Caco-2 cell line results in electrical properties often characterised by a high TEER, that more closely resemble those of the colonic epithelium rather than the lower resistance of the small intestinal absorptive epithelium (35). For this reason, Caco-2 cell lines exhibiting lower TEER values are considered to be more representative of the electrical permeability of the small intestinal epithelium *in vivo*, provided that paracellular solute permeability remains low.

As confirmed by the statistical analysis, TEER values reached a plateau around day 15 in culture in all the laboratories, although the absolute values varied with the cell line. However, even if substantial differences were observed in the maximal values achieved during differentiation in the four cell lines, the rank order was similar in the six laboratories using either DMEM/FCS-sym or DMEM/ITS/L, with Caco-2/TC7 always displaying lower values. TEER measurements were easy to perform, were not influenced by the medium composition and, in general, gave good within-laboratory reproducibility. This parameter can therefore be recommended for comparison purposes between clones and treatments in the same laboratory and among laboratories.

The Ap to Bl flux of [<sup>3</sup>H] mannitol (MAN), was used to determine the permeability of the paracellular pathway of filter-grown cells. This parameter was shown to be influenced by the medium composition — the tight-junction permeability to small solutes of cells maintained in the synthetic medium DMEM/ITS/L-asym was always higher and less reproducible than for cells in DMEM/FCS-sym medium. In addition, MAN appeared to be less influenced by the cell type than TEER. The disadvantages of this parameter lie in the use of radio-labelled material and in the instability of [<sup>3</sup>H] mannitol during storage. [<sup>14</sup>C] mannitol, although a

more stable alternative, is also more expensive. Since the measure of MAN is more stable than that of TEER, as it is not influenced by ion fluxes (25, 36), it can be recommended as a good indicator of the effective permeability of the epithelial cell monolayer to small solutes.

The differentiation marker, AP activity, was shown to be influenced by medium composition and cell type. Measured by all the participants under standardised experimental conditions, it was shown to be a reliable and reproducible marker among the laboratories. As was found with TEER, AP ranking in the parental Caco-2 cells and in the three clonal lines was the same in the six laboratories, in both DMEM/FCS-sym and DMEM/ITS/L-asym. AP activity was relatively stable from day 15 onwards in DMEM/FCS-sym medium, while in DMEM/ITS/L-asym, a tendency for a further increase in activity from day 15 to day 21 was observed in certain cell line-laboratory combinations. This may reflect an incomplete differentiation of the cells in synthetic compared to serum-containing medium.

The results of this study permit the following general conclusions to be drawn.

1. *Cell lines.* Taking into account the statistical analysis of the results of the endpoints measured, Caco-2/TC7 cells performed better than the other three cell lines, both in terms of reproducibility and performance. They achieved the best level of differentiation, as shown by the highest AP activities, together with low TEER and MAN values, thus more closely resembling the barrier properties of the small intestine *in vivo*. Therefore, Caco-2/TC7 cells should be recommended as the *in vitro* cell model which best represents the barrier properties of the small intestinal epithelium. To substantiate these conclusions further, it has previously been observed that this clonal line appears almost unresponsive to some growth stimuli, suggesting a highly differentiated state (24). In addition, it was shown that Caco-2/TC7 cells have an enterocyte-like morphology (basal localisation of nuclei, regular microvilli, well-developed tight junctions, etc.) and express higher levels of CYP 3A than do the Caco-2/parental cells and the other two clonal cell lines (24).

Conversely, Caco-2/parental and Caco-2/15 cells expressed low levels of alkaline phosphatase, indicating a lower level of differentiation. Moreover, Caco-2/15 cells showed very high TEER values, more similar to that of the colonic epithelium, while Caco-2/AQ cells gave variable results.

2. *Culture medium.* All the cell lines exhibited a better performance (in terms of variability and/or response level), when maintained in DMEM/FCS-sym than in the defined medium,

DMEM/ITS-L. This may be partially accounted for by the lack of standardisation and experience in preparing the defined medium. However, other factors related to the composition of the defined medium cannot be ruled out and require further investigation. In conclusion, the use of the defined medium DMEM/ITS-L, although not optimal, has shown to be promising, so further studies should be encouraged.

3. *Culture period.* A culture period of 15 days results in stable levels for the three parameters under investigation, and further prolongation of the culturing period appears to be of little benefit. However, when maintained in DMEM/ITS/L-*asym* medium, some evidence pointed to an increase in AP activity beyond day 15, which may suggest incomplete or slower differentiation of the cells under these conditions. In addition, other differentiation markers, such as some transport and metabolic activities, have been shown to increase up to day 21 or beyond (9). Therefore, the 15-day time limit may be sufficient for the development of permeability properties, but not for full epithelial differentiation.
4. *Endpoints and parameters.* The three parameters selected in this study have shown good within-laboratory reproducibility under the standardised experimental conditions used. In addition, they have proven to be sensitive enough to discriminate among the different cell lines and media compositions. With respect to the assessment of barrier integrity, TEER has shown to be a simple and rapid indicator of this epithelial property, although the large variability in the absolute TEER values among the different cell lines is likely to reflect structural differences in the molecular composition of tight junctions, rather than real differences in permeability to small solutes (36, 37). As an indicator of barrier permeability, MAN passage has proven to be better than TEER, especially when comparing different cell lines, although it is more time-consuming to measure and it requires the use of a radioactive probe. The reliability and usefulness of AP activity as a straightforward and easily measurable differentiation marker was confirmed. Clearly, one endpoint is not enough to characterise a complex process such as intestinal differentiation, but, in practical terms, it is important to establish a limited number of endpoints relevant to the standardisation of the cellular model to be optimised.

In conclusion, this study has shown that only the use of standardised protocols in Caco-2 cell investigations permits a meaningful comparison of the results obtained in different laboratories. The exist-

ing literature on Caco-2 cells suffers from a lack of standardisation of experimental designs and protocols, with the consequence that general conclusions on the adequacy of these cells for different types of investigations are often difficult to draw. This aspect is especially relevant in the discussion on whether *in vitro* tests can be reliable substitutes of *in vivo* studies. In view of a reduction in the use of animal experimentation in toxicology and pharmacology, it is therefore particularly urgent to progress the available *in vitro* models to the validation stage. Due to the widespread and ever increasing use of the human intestinal Caco-2 cell lines in several fields of investigation, it is hoped that this preliminary investigation could lay the foundations for a future follow-up study, rapidly leading to the validation process and the subsequent acceptance at the regulatory level of this model for intestinal absorption and transport studies.

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