
Design, development and validation of a compact transcutaneous fluorescence sensor for non-invasive assessment of gut function

Author Elena Monfort-Sanchez **Supervisors** Dr. Alex Thompson¹
Submitted 16th May 2024 Dr. James Avery^{1,2}
Prof. Ara Darzi¹

¹Department of Surgery and Cancer, Imperial College London, UK.

²School of Electronic and Electrical Engineering, University of Leeds, UK.

A thesis submitted in fulfilment of the requirements for the degree of *Philosophiae Doctor*

The Hamlyn Centre, Institute of Global Health Innovation
Department of Surgery and Cancer
Imperial College London

The Hamlyn Centre, Institute of Global Health Innovation,
Department of Surgery and Cancer,
Imperial College London,
Bessemer Building,
South Kensington,
SW7 2AZ

Elena Monfort-Sanchez © 2024

To my grandparents, Milagros and Juan.

Declaration

All the work here presented in this thesis is my own with the following exception:

- Data collection for the clinical study with healthy volunteers discussed in chapter 3 was performed by Dr Jonathan Gan.
- Assembly of the miniaturisation version of the optical sensor discussed in chapter 4 was performed in collaboration with Dr Nilanjan Mandal.
- Data collection, including fluorescence signals, urine sample and morphometry data, for the undernourished participants discussed in chapter 5 was performed by a number of colleagues from St. Augustine clinic in Lusaka, Zambia.
- Protocols for the culture of cell monolayers were provided and performed in collaboration with Dr Tafhima Haider.

Copyright

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

Firstly, I would like to thank Alex Thompson and James Avery, my supervisors, whose guidance, support, and expertise were invaluable throughout the course of this research. I would like to acknowledge their encouragement and insightful feedback throughout these years, which has been instrumental in the progress of this thesis. I would also like to thank Prof Ara Darzi, for giving me the opportunity to work in this project. Additionally, I would like to acknowledge the UK Medical Research Council (MRC) and National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC) for funding my Ph.D. To the GI Tools members, especially to Paul Kelly, Mulima Mwiinga and Rose Banda for their time and patient to learn - remotely - how to use the sensor presented in Chapter 5. Being in different countries definitely made it even more challenging. Final academic thanks should also go to Claire Bourke, Tafhima Haider and Despoina Chrysostomou for their invaluable guidance on the cell work presented in Chapter 7.

I would also like to thank all my colleagues both in Paterson and Bessemer buildings from Imperial with whom I worked closely. In particular, I should mention Jonathan Gan, Mark Runcinman, Amir Amirhosein, Yingwei Hou and Nilanjan Mandal, for their time, continuous support and useful advice during this long journey. Of course, a special mention to Mark Runcinman and Nilanjan Mandal for their constant supply of food, drinks and good time over the course of my PhD. I also want to thank all my friends, both past and present. A special mention to Olivia and Cristina, who very often provided an opportunity to step outside of the lab and enjoy the conversations, the fresh air and the sun (rarely). Most importantly, I would like to thank my family - from UK and Spain, for their constant and invaluable emotional support. To my parents, my sister and Alex, for being there every single time throughout all these years. They are my biggest supporters and I will forever be grateful.

Abstract

The integrity of the intestinal barrier is a significant contributor to various pathophysiological conditions, including malnutrition. Abnormalities in the structure and function of the GI tract can lead to increased intestinal permeability, inflammation and severe nutrient malabsorption. Despite the importance of gut function, current techniques to assess intestinal permeability are costly, invasive, unreliable and/or difficult to perform in certain populations.

To address this, this thesis describes the development and validation of an optical sensing technology – transcutaneous fluorescence spectroscopy – to improve non-invasive assessment of gut permeability. This technique involves the oral administration of fluorescein and the following measurement of fluorescence signals non-invasively through the skin. The thesis first reports development of computational algorithms to process transcutaneous fluorescence signals. These algorithms provided successful classification of fluorescence signals from different gut permeability conditions induced in healthy volunteers.

To further optimise this technology to facilitate more widespread clinical adoption, subsequent work focused on developing a portable optical sensor. This system utilises a fibre-optic probe attached to a finger clip for light delivery/collection. In vivo validation of this device demonstrated non-invasive detection of fluorescein's permeation under different conditions, further showcasing potential for clinical use. A subsequent clinical study demonstrated the capability to distinguish healthy and undernourished participants, and revealed correlation of data with existing, clinically-approved tests.

Finally, an in vitro study was performed to evaluate permeation of fluorescein (and other fluorophores) through cell monolayer models of the intestinal barrier. This study further validated fluorescein as a marker of impaired gut barrier function and indicated that it could provide a low-cost alternative to larger molecules (such as FITC-dextran) for assessment of epithelial permeability in cellular models.

Overall, this thesis reports the development and validation of a portable fluorescence sensing technology for clinical assessment of gut permeability and the implementation of computational techniques for rapid clinical diagnostics/assessments.

Contents

1	Thesis Overview	42
1.1	Publications	44
2	Clinical Motivation	46
2.1	Gastrointestinal Permeability	47
2.1.1	Gastrointestinal Epithelium: a multifunctional barrier	47
2.1.2	Molecular Transportation across the Gastrointestinal Barrier	48
2.1.3	Gastrointestinal Pathologies - the role of gut permeability	50
2.1.3.1	Undernutrition	51
2.2	Current diagnostic landscape for the assessment of gut permeability	53
2.2.1	Endoscopic biopsies	53
2.2.1.1	Confocal endomicroscopy	55
2.2.2	Permeability tests	55
2.2.2.1	Lactulose:Mannitol permeability test	55
2.2.2.2	Lactulose:Rhamnose permeability test	56
2.3	The need for better assessment of gut barrier integrity	57
3	Fluorescence Spectroscopy for the assessment of gut permeability	59
3.1	Fluorescence Spectroscopy	60
3.1.1	Photoluminescence	60
3.1.2	Properties of fluorescence molecules	62
3.1.3	Endogenous and exogenous fluorophores	62
3.1.4	Transcutaneous fluorescence spectroscopy	64
3.2	Applications of fluorescence spectroscopy for the assessment of the gut barrier	67
4	Automatic processing and analysis of fluorescence spectroscopy data - Proof-of-concept	69
4.1	Introduction	70
4.1.1	Aims	71
4.2	Methods	71
4.2.1	Fibre-optic spectrometer	71
4.2.2	Clinical trial measurements	73
4.2.3	Investigation of different computational approaches for data analysis	74
4.2.3.1	Automatic peak selection	76

4.2.3.2	Exponential decay fitting	77
4.2.3.3	Phasor analysis	79
4.2.4	Statistical analysis - comparison between induced permeability stages	80
4.3	Results	81
4.3.1	Signal processing and computational parameter extraction	81
4.3.1.1	Automatic Peak Selection	81
4.3.1.2	Exponential decay fitting	88
4.3.1.3	Phasor analysis	97
4.3.2	Signal analysis: comparison between different gut permeability stages	99
4.3.2.1	Fluorescence intensity vs time curves	99
4.3.2.2	Classification of different gut conditions - Automatic Peak Selection	102
4.3.2.3	Classification of different gut conditions - Exponential decay fitting	105
4.3.2.4	Classification of different gut conditions - Phasor analysis	105
4.4	Conclusion	109
5	Fibre-optic fluorescence spectroscopy sensor - development and validation	111
5.1	Introduction	112
5.2	Methods	113
5.2.1	Benchtop System – control system	113
5.2.2	Instrument development	113
5.2.2.1	Compact fibre-optic fluorescence sensor – optical set-up	113
5.2.2.2	Compact fibre-optic fluorescence sensor – detailed description of the custom fibre-probe	116
5.2.2.3	Compact fibre-optic fluorescence sensor – electronic configuration	118
5.2.2.4	Calculation of normalized fluorescence values	121
5.2.2.5	In-house written Control Software	122
5.2.2.6	Safety considerations	124
5.2.3	Experimental procedures - validation	125
5.2.3.1	Fluorescence samples measurement – laboratory validation	125
5.2.3.2	Human measurement – in vivo validation	127
5.2.4	Data Analysis	129
5.3	Results	130
5.3.1	Laboratory validation	130
5.3.1.1	Fluorescent samples measurements	130
5.3.1.2	Temperature and optical power measurements	132
5.3.1.3	Motion Artifact analysis	132

5.3.2	In vivo validation	134
5.3.2.1	Fluorescence data Analysis – Stage 1	134
5.3.2.2	Compact sensor characterization – Impact of contrast agent dose – Stage 2	139
5.3.2.3	Compact sensor characterization – Impact of skin tone – Stage 3	144
5.3.3	Miniaturization	146
5.3.3.1	PCB design and assembly	146
5.4	Conclusion	150
6	Clinical validation of a compact fluorescence sensor: monitoring gut permeability in healthy and undernourished populations	151
6.1	Introduction	152
6.2	Methods	153
6.2.1	Compact transcutaneous fluorescence sensor	153
6.2.2	Experimental procedures	154
6.2.2.1	Calibration	154
6.2.2.2	Clinical trial measurements	157
6.2.2.3	Permeability assays - sugar molecules	160
6.2.2.4	Endoscopic biopsies	161
6.2.3	Statistical analysis	162
6.3	Results	163
6.3.1	Compact fiber-optic sensor calibration	163
6.3.2	Fluorescence intensity vs time - Healthy study	167
6.3.2.1	Fluorescence signal analysis	167
6.3.2.2	Lactulose:Mannitol correlation	170
6.3.2.3	Summary	172
6.3.3	Fluorescence intensity vs time - Undernutrition study	173
6.3.3.1	Fluorescence signal analysis	173
6.3.3.2	Lactulose:Rhamnose correlation	175
6.3.3.3	Morphometry analysis	178
6.3.3.4	Summary	182
6.3.4	Comparison between Group 1 (Healthy) and Group 2 (Undernourished) datasets	183
6.3.4.1	Compact sensors - Photodiode signals	183
6.3.4.2	Compact sensors - Fluorescence intensity signals	185
6.3.4.3	Sugar molecules permeability tests	189
6.3.4.4	Advanced analysis for automatic classification of fluorescence datasets	194

6.4	Conclusion	197
7	In vitro investigation of alternative fluorescence contrast agents in 2D epithelial cell monolayer models	199
7.1	Introduction	200
7.2	Methods	201
7.2.1	Gut barrier model in cell monolayer cultures	201
7.2.1.1	Cell types: epithelial cell model	202
7.2.2	Disruption of tight junctions	203
7.2.3	Fluorescence contrast agents	204
7.2.4	Experimental procedures	204
7.2.4.1	Cell monolayer culture	205
7.2.4.2	Disruptor optimisation	207
7.2.4.3	Dye optimisation	210
7.2.4.4	Transwell insert - control permeability	213
7.2.4.5	Permeability assay	213
7.2.5	Protocols	214
7.3	Results	219
7.3.1	Disruptor optimisation	219
7.3.2	Dye optimisation	220
7.3.3	Transwell insert - control permeability	222
7.3.4	Permeability assay	224
7.4	Conclusion	231
8	Conclusion and future work	233
8.1	Summary	234
8.2	Computational analysis of fluorescence spectroscopy data	234
8.2.1	Algorithm development for automatic processing and analysis of fluorescence spectroscopy data – proof-of-concept	234
8.3	Development and characterisation of a compact fluorescence sensor	237
8.4	Clinical validation of a compact fluorescence sensor	239
8.4.1	Monitoring gut permeability in healthy and undernourished populations – a comparative study	239
8.5	Alternative fluorophores for gut permeability assessment	241
8.5.1	In vitro investigation of potential fluorescence contrast agents in 2D MDCK monolayer models	241
8.6	Final remarks	243
	References	244

List of Figures

2.1	(A) Illustration of the structure of the different Intestinal epithelial cells. The intestinal epithelial stem (IES) cells control the renewal of the epithelial barrier. New epithelial cells migrate from the bottom of the crypt up to the crypt–villus axis (dotted arrow). Secretory goblet cells and Paneth cells secrete mucus and antimicrobial proteins to protect the epithelial surface. (B) Key layers of the intestinal barrier. The mucus layer also contains a proportion of the intestinal microbiota. The epithelial layer contains many specialized cell types, including enterocytes and Paneth cells, among others. The cells in the epithelium are connected by transmembrane protein complexes, including tight junctions, creating a physical barrier between the lumen and the lamina propria. The deeper layer, the lamina propria, is a connective tissue layer that contains immune cells, blood vessels and lymphatic vessels.	48
2.2	Illustration of the paracellular and transcellular transport of molecules in a (A) healthy and (B) compromised epithelial barrier. The formation of a polarised cell monolayer is characterised by the presence of an apical compartment, in contact with the intestinal lumen, and a basolateral compartment, in contact with the connective tissue. The cells are linked together through tight junction complexes. The transcellular pathway is an active transport mediated by transporters or endocytosis-exocytosis. The paracellular pathway, on the contrary, is a passive transport mediated by the pore or leak pathways. In a disrupted intestinal barrier (B) , integrity of the tight junctions is compromised increasing the inter-cellular spaces. In more severe conditions, a third pathway, the unrestricted pathway, is generated. In this case, large molecules, including bacteria, can freely traverse the barrier.	49
2.3	Image showing the morphometry measurements: villus height (A) , epithelium height (B) , crypt depth (C) and epithelial surface area (D) , as well as other substructures, on a chicken model. Image taken from Alshamy et al. [[100]].	54
3.1	(A) Jablonski diagram illustrating the energy levels transitions often found in photoluminescence processes: fluorescence and phosphorescence. Single (S0, S1, S2) and triplet (T1) states are represented. (B) Singlet vs Triplet state representation.	60

3.2	Illustration of the basic configuration of the steady state fluorescence spectroscopy. Inside illustration shows the main transition occurring when a fluorophore is excited by a specific wavelength.	66
4.1	Portable fibre-optic fluorescence spectrometer used to investigate non-invasive assessment of intestinal permeability. Schematic diagram shows the layout of the optical system. Insets show arrangements of optical fibres at the distal and proximal ends of the bifurcated fibre probe (excitation fibre – blue; collection fibres – yellow). Dotted red box indicates that the 785 nm light source was not used in this study. Photographs on the right show (<i>top</i>) the 3D-printed wearable finger clip that was used to secure the tip of the optical fibre probe in contact with the skin during measurements and (<i>bottom</i>) the trolley-mounted spectrometer. ND – neutral density. Figure amended from Maurice et al. [90] under the terms of the Creative Commons CC BY license.	72
4.2	Example of the fluorescence vs time profile after oral administration of a 500mg dose of fluorescein. The fluorescence signal is characterised by three phases: <i>phase 1</i> , where the kinetics is primarily governed by the permeation/absorption of the fluorescein; <i>phase 2</i> occurs at the time (Peak Time) when the maximum plasma concentration (Peak Intensity) is reached; finally, <i>phase 3</i> starts when the fluorescein plasma clearance is predominant. The parameters extracted from the fluorescence vs time curves, relevant to the pharmacokinetics of the fluorescein, are depicted in the graph: Peak Intensity, Peak Time, Peak AUC and Slope.	75
4.3	Algorithm development workflow. (A) Example of a fluorescence intensity as a function of time after ingestion of fluorescein for the fluorescein only (solid line) and fluorescein + sugar (dotted line) measurements. (B) Workflow for Method 1 for the analysis of the data. Peak Time and Peak Intensity values are extracted manually from the raw data. The rest of the parameters are calculated computationally using MATLAB software. (C) Workflow for Method 2 for the analysis of the data. Raw data is first filtered computationally using MATLAB software. Peak Time and Peak Intensity values are then extracted manually from the filtered data. The rest of the parameters are calculated computationally using again MATLAB software. (D) Workflow for Method 3 for the analysis of the data. Again, raw data is initially filtered. All parameters, including Peak Time and Peak Intensity are then extracted and calculated computationally from the filtered data using MATLAB software . . .	77
4.4	Illustration of the workflow followed to correct the fluorescence intensity signals using the fitting algorithm approach. The parameters extracted from the fitting function are then used to correct for the fluorescence decay rate from the collected signal.	79

4.5	Parameters extracted from the fluorescence vs time curves. (A) Fluorescence signals from Participant 5 and 11 for the fluorescence-only study. In this case, both participants show a typical time-resolved fluorescence curve, with a single peak followed by a decay towards the baseline. (B) Fluorescence signals from Participants 5 and 11 for the fluorescence + sugar study. In this case, both participants exhibit a “double peak phenomenon” (an effect observed in certain drugs [226] with biphasic absorption behavior). Parameters – Peak Time, Peak Intensity and Peak AUC – extracted from the signals are shown. The green (A) and orange (B) regions represent the area under the curve (AUC) up until the first peak, which determines the values of the Peak AUC parameter.	82
4.6	Comparison of different median filter windows using the Fluorescence vs time curves for Participant 11. Raw data is presented in blue and filtered data is presented in red. (A) Median filter with a window of 5. Noise within the raw data (<i>blue</i>), manifested as rapid and discontinuous changes in amplitude, is still present in the filtered data (<i>red</i>). (B) Median filter with a window of 10. Noise is effectively removed from the raw data(<i>blue</i>), while preserving the profile of the curve. (C) Medial filter with a window of 15. Signal distortion is produced due to excessive attenuation, leading to information loss.	84
4.7	Exponential decay approaches: examples of fluorescence – only signals from Participant 7 and 11. (A) Single (<i>1-term</i>) exponential decay model. The fluorescence – only data used for the fitting is represented in blue and the starting point is determined by the Peak Time, extracted from Method 3 (see Section 4.2.3.1). From the predicted decay function ($I(t)$), in red, the parameters I_0 and b can be extracted and will be used to correct for the elimination of the fluorescein in the body. The performance of the fitting model is assessed by the R^2 and RMSE values, also shown in the graphs. (B) Double (<i>2-term</i>) exponential decay model. As before, the fluorescence data used for the fitting is represented in blue and the starting point is determined by the Peak Time from Method 3. In this case, parameters a , b , c , and d are extracted from the predicted decay function ($I(t)$), in red, and will be used to correct for the elimination of the fluorescein in the body. The performance of the fitting model is also assessed by the R^2 and RMSE values.	89
4.8	Single (<i>1-term</i>) exponential decay fitting applied to the Fluorescence vs time curves for all 11 participants under two experimental conditions: fluorescein solution only (<i>left</i>) and fluorescein solution + sugar (<i>right</i>). Data points used for the fitting approaches are represented in <i>blue</i> and the resulting fitting functions are illustrated in <i>red</i>	91

4.9	Exponential decay fitting applied to the Fluorescence vs time curves for Participants 5, 6 and 8, using the new suggested Peak Times. Data points used for the fitting approaches are represented in blue and the resulting fitting functions are illustrated in red.	92
4.10	Exponential decay correction using the predicted parameters extracted from 1-term and 2-term approaches. (A) Fluorescence signals from Participants 3 and 11 (left graphs) and the fitting functions (in red) predicted by 1-term (<i>top</i>) and 2-term (<i>bottom</i>) approaches. In this case, both exponential decay fitting approaches provided R^2 values > 0.900 . This determined a good prediction of the clearance of fluorescein, recovering the data on the clearance phase to a plateau profile (right). (B) Fluorescence signals from Participants 8 (left graphs) and the fitting functions (in red) predicted by 1-term (<i>top</i>) and 2-term (<i>bottom</i>) approaches. In this case, both exponential decay fitting approaches provided R^2 values < 0.800 . This determined an inaccurate prediction of the clearance of fluorescein (see right graphs).	95
4.11	Impact of the Peak Time values on the corrected fluorescence signals. (<i>top</i>) Double exponential decay fitting (red) applied to the blue data points from the fluorescence signal (black). The starting point for the fitting algorithm is the original Peak Time value for Participant 5 (27 minutes). The corrected fluorescence data is illustrated on the right, with a maximum peak intensity of 10.772. (<i>bottom</i>) Double exponential decay fitting using in this case a modified peak time of 20 minutes. In this case, the corrected fluorescence data, illustrated on the right, has a maximum peak intensity of 0.0165.	96
4.12	Mean and STD fluorescence signals after the correction for the elimination of fluorescein from the body is applied. Mean fluorescence-only (blue) and fluorescence + sugar (red) signals after applying the correction decay from the 1-term model (left) and 2-term model (right).	97
4.13	Fluorescence vs time curves for all 11 participants under two experimental conditions: fluorescein solution only (blue) and fluorescein solution + sugar (red). Fluorescence signals for each condition were collected on different days using the spectroscopy system displayed in Figure 4.1 for a total period of 180 minutes.	100
4.14	Mean fluorescence intensity as a function of time after ingestion of fluorescein for the fluorescein only (solid blue line) and fluorescein + sugar (dotted red line) measurements. The blue and red lines represent mean values calculated across all 11 participants. The shaded blue and red regions represent ± 1 standard deviation from the mean. Peak Time and Peak Intensity values calculated from the mean fluorescence curves are also displayed (not to be confused with the average Peak Time and Peak Intensity values calculated amongst all 11 participants, shown in Tables 4.2, 4.3) and 4.4.	101

4.15	Comparison of the parameters extracted from the fluorescence vs time curves. (left to right) Scatter-Box plots showing the parameter values calculated using Method 1, Method 2 and Method 3. (top to bottom) Parameters extracted from the fluorescence curves – Peak Time, Peak Intensity, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time. Box plots also show median values (red lines), 25 th and 75 th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for the fluorescence only study (blue) and fluorescein + sugar study (red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25 th percentile or above the 75 th percentile by more than 1.5 times the interquartile range. All calculations (e.g. of means, medians, etc.) and all statistical tests were performed based on the data points from all 11 participants (i.e. including outliers) in all cases.	103
4.16	Phasor components extract from the fluorescence signals. (A) Phasor plot with the Real (g) and Imaginary (s) components of each participant for the fluorescence-only (blue) and fluorescence + sugar (red) studies. Mean values, as well as the 95% confident interval (ellipses), are also shown. (B) Vector plot of the individual phasor components of each participant. Fluorescence-only phasor components are represented in blue and fluorescence + sugar phasor components are represented in red. The black arrow illustrates the shift of the data from study day 1 (fluorescence -only) and day 2 (fluorescence + sugar).	106
4.17	The decision boundary generated by the SVM algorithm implemented with the phasor components extracted from the fluorescence signals. SVM support vector are also illustrated in the graph with circles.	107
5.1	Compact fluorescence sensor for transcutaneous assessment of gut function. (A) Schematic diagram of the compact fluorescence sensor; (B) Custom fibre probe diagram showing distal fibre arrangement (left) and full bundle (right). (C) Excitation/emission/sensitivity spectra of optical components (including fluorescent contrast agent) used in the compact fluorescence sensor; data extracted from component datasheets. (D) Photograph of compact sensor deployed in a clinical environment. The laptop shows the user interface developed for collection and pre-processing of data. The interface can be seen in more detail in Section 5.2.2.5 - Fig. 5.6. A 3D printed finger clip is shown next to the fibre tip. A close-up of the distal tip of the optical fibre probe is shown in the insert (red box).	115

- 5.2 Light suppression comparison between single and dual filter configuration. Graphs show spectra collected using a FLAME spectrometer (Ocean Insight) when LED excitation light (465 nm central emission wavelength) was directed through long pass and short pass filters (with cut-off frequency at 500 nm) into an optical fibre probe connected to the spectrometer. **(A)** Spectra acquired for single (*left*) and dual (*right*) filter configurations with 1 ms integration time. Inset graphs show close-up views of the spectra. **(B)** Spectra acquired for single (*left*) and dual (*right*) filter configurations with 20 ms integration time. For both integration times, the residual LED signal is considerably lower when using the dual filter configuration as compared to the single filter configuration. 116
- 5.3 Diagrams illustrating the arrangement of fibres at the distal and proximal ends of the custom optical fibre probe: **(A)** shows the front and cross section fibre configuration at distal end; **(B)** displays the end view of the proximal ends for the excitation branch (red fibre labelled as LED) and collection branches (labelled as PD_1 and PD_2). 117
- 5.4 Characteristic signals obtained in vivo using the compact fluorescence sensor. **(A)** Example raw data (after amplification) collected with the compact sensor in a clinical experiment lasting approximately 100 minutes. **(B)** Close-up of data collected in a single duty cycle (of 10 seconds) from data displayed in (A) (as indicated by the arrow in panel A). graphs show signals from both the Fluorescence Photodiode (blue) and Backscatter Photodiode (red). ON and OFF window regions are displayed in (B). These regions were used to extract the photodiode output voltages for calculation of the normalized fluorescence signal. 118
- 5.5 Electronic configuration for the compact sensor. **(A)** Circuit design in LTSpice to characterize the starting point of the electronic gains suitable to amplify the photodiodes signal. The values R_f , R_2 and R_3 are then used as a starting point to design the next iterations of the electronic circuit. **(B)** Final version of the electronic configuration of the compact sensor. Values of the components used in the final version of the sensor are also depicted. 120
- 5.6 The LabView user interface developed for collection and pre-processing of data. **(A)** Main tab of the LabView interface showing the raw voltage data collected by the sensor photodiodes (upper graph; PD0 – Backscatter Photodiode; PD1 – Fluorescence Photodiode), the normalized fluorescence intensity data (lower graph), and the main sensor/acquisition controls. **(B)** Secondary interface tab with additional information (including mean 'Off' and 'On' data for each photodiode). . . . 123

- 5.7 *In vitro* experimental setup. **(A)** Photograph of experimental setup. The sensor (placed below the laptop) collects the fluorescence intensity of fluorescein solutions in transparent thermoplastic containers. Thermoplastic containers are displayed on the right-hand side of the image and contain aqueous fluorescein solutions at concentrations of 0-0.0017 mg/ml. Container with 0 mg/ml fluorescein (i.e., baseline containing only water) is not visible in the image. This container is located inside a black box (marked in red), which was used to minimize background signals. **(B)** Diagram of the inside of the black box (marked in red in panel A) used to collect fluorescence data. The fluorescein solution is attached to the inside of the box using 3D printed supports to maintain a constant position during measurements. The finger clip (including the optical fibre probe) is clamped to the fluorescein sample. The spring-loaded mechanism of the finger clip allows a secure and constant position to be maintained throughout the collection time. **(C)** Experimental set up for Motion artifact analysis. The sensor is attached to the fingertip of one of the participants and an accelerator is secured on top of the same hand. The sensor and the accelerator were subjected to three hand-motion scenarios: static (no motion), low and fast movements. The accelerometer collected data from the x, y and z-axes. 126
- 5.8 Laboratory validation of compact fluorescence sensor (against benchtop system) and safety evaluation. **(A)** Normalized fluorescence intensity as a function of fluorescein concentration (in aqueous solutions) for the compact sensor (*blue, left y-axis*) and benchtop system (*orange, right y-axis*). Concentration range: 0-0.0017 mg/ml. Baseline levels (signals from a concentration of 0 mg/ml fluorescein) for each system were subtracted from all measurements (hence, the first datapoint occurs at the origin (0,0) for both devices). **(B)** Temperature measurements collected over a time period of 3 hours for two different locations: surface of the LED (left), tip of the fibre probe (right). Temperature measurements (at the surface of the LED) over three continuous duty cycle are presented in D. **(C)** Optical power measurements collected over a time period of 3 hours for the same locations: surface of the LED (*left*), tip of the fibre probe (*right*). Background optical power (from ambient light) was measured and subtracted from the recorded signals. **(D)** Temperature measurement at the surface of the LED. (*left*) Temperature as a function of time for data collected over a time period of 180 minutes. (*right*) Close up of the region highlighted by the red box in the left graph. This region represents three complete LED duty cycles (illustrated with red arrows), which include 7 seconds of LED on and 3 seconds of LED off in all three cycles. 131

5.9	Motion Artifact analysis: comparison of different hand-motion scenarios and compact sensor stability for a period of 50 minutes. (A) Displacement along the x, y and z axes derived from the accelerometer dataset. The three motion scenarios: static, slow and fast movements, are visually identifiable, as the frequency and displacement patterns differ between scenarios. (B) Sensor output voltages from both Photodiodes (PD_F : Fluorescence Photodiodes and PD_{BS} : Backscattered Photodiodes).	133
5.10	In vivo validation measurements in healthy volunteers. (A) . Examples of Scaled fluorescence intensity vs. time curves from compact fluorescence sensor (<i>blue</i>) and benchtop system (<i>orange</i>) for participants 3 and 4. Participant 3 shows a typical time-resolved fluorescence curve, with a single peak followed by a decay towards the baseline. Participant 4, on the other hand, exhibits a “double peak phenomenon” (an effect observed in certain drugs [226] with biphasic absorption behaviour). (B) The mean and standard deviation (STD) of the fluorescence intensity vs. time curves for both the compact sensor (blue) and benchtop system (orange). The individual scaled fluorescence intensity vs. time curves for participants 1-8 are presented in Fig. 5.11. (C) The mean area under the curve (AUC) calculated for both systems (for participants 1-8). Error bars represent upper and lower bounds on the AUC values calculated by assessing the AUC for the mean curves ± 1 STD (i.e., the AUC values corresponding to the upper and lower bounds of the shaded regions in B). Total AUC values for each participant and for the mean curves are presented in Table 5.3.	135
5.11	Individual scaled fluorescence intensity vs. time curves for participants 1-8. Benchtop system – orange; compact sensor – blue. Signals were recorded for a total of 180 minutes, with the exception of participants 1 and 2, where data collection issues necessitated early termination of experiments (after 135 and 90 minutes respectively). The presented fluorescence vs. time curves are scaled to their maximum intensity (from 0 – 1) in order to address the different absolute values produced by the compact sensor and benchtop system (due to differences in the detection electronics used and the corresponding outputs). This facilitated investigation of correlation between datasets collected with the two devices.	136

- 5.12 Characterization of compact sensor under different fluorescein dose conditions. **(A)** Time-resolved normalized fluorescence signals for participants 1-8 (left; 500 mg fluorescein in 100 ml water) and participants 9-14 (right; 200 mg fluorescein in 100 ml water). Participants 1 and 2 present shorter signals (135 and 90 minutes respectively) due to data collection issues that necessitated early termination of experiments. **(B)** Mean ($\pm$ STD) normalized fluorescence vs. time curves for both dose conditions: 500 mg (blue), 200 mg (orange). The irregularities/discontinuities observed at 90 and 135 minutes on the mean normalized fluorescence signal for the 500 mg dose are a result of the shorter data collection times for participants 1 and 2. **(C)** Mean peak (maximum) intensity values extracted from the normalized intensity curves shown in panel A. Note that the maximum intensity values in panel C do not correlate to the maximum values observed on the curves shown in panel B due to variability in the time at which peak intensities were observed in each participant (see panel A). That is, the values reported in panel C represent the averages of the maximum intensity values observed in each curve in panel A. Thus, any effect of peak time is removed/ignored in the average calculation in panel C (unlike in panel B, where varying peak time leads to changes in the location and intensity of the curve's peak). A statistically significant difference was observed between the 500 mg and 200 mg peak intensity values. When a correction factor (of 2.5) was applied to the 200 mg peak intensity ("*Corrected 200 mg*"; green bar), no statistically significant difference was observed when compared against the 500 mg dose, indicating an approximately linear relationship between dose and peak intensity. * – p value < 0.05; ns – not significant. 140
- 5.13 Peak Time, Total AUC and Normalized Peak Intensity values calculated from the compact sensor datasets for all participants (P1–14). Values for participants 3–14 were extracted from the full 180 minutes of collected data. Due to early termination of data collection (explained above), parameters for participants 1 and 2 (indicated with an *) were extracted from 135 and 90 minutes of data respectively. On each graph, data is classified according to contrast agent dose (including the "*Corrected 200 mg*" data, where data collected at 200 mg was multiplied by a correction factor of 2.5 to allow comparison to data collected at 500 mg). Bars are color-coded based on the Fitzpatrick skin tone type for each participant (see legend). The numbers of participants in each Fitzpatrick group are shown in the legend. Dotted lines represent the values from the mean fluorescence vs. time curves (presented in Fig. 5.12B) for each dose condition. **(A)** Peak Time parameter representing the time (in minutes) at which the signal reaches maximum intensity. **(B)** Total AUC. **(C)** Normalized Peak Intensity representing the maximum fluorescence intensity value for each dataset. 141

- 5.14 Characterization of benchtop system under different fluorescein dose conditions. **(A)** Time-resolved normalized fluorescence signals for participants 1-8 (*left*; 500 mg fluorescein in 100 ml water) and participants 9-14 (*right*; 200 mg fluorescein in 100 ml water). Participant 1 and 2 present shorter signals (135 and 90 minutes respectively) due to data collection issues that necessitated early termination of experiments. **(B)** Mean ($\pm$ STD) normalized fluorescence vs. time curves for both dose conditions: 500 mg (blue), 200 mg (orange). The irregularities/discontinuities observed at 90 and 135 minutes on the mean normalized fluorescence signal for the 500 mg dose are a result of the shorter data collection times for participants 1 and 2. **(C)** Mean Peak (maximum) intensity values extracted from the normalized intensity curves shown in panel A. A statistically significant difference was observed between the 500 mg and 200 mg peak intensity values (p value = 0.0197). When a correction factor (of 2.5) was applied to the 200 mg peak intensity ("*Corrected 200 mg*"; green bar), no statistically significant difference was observed when compared against the 500 mg dose (p value = 0.6432), indicating an approximately linear relationship between dose and peak intensity. * – p value < 0.05; ns – not significant. 142
- 5.15 Peak Time, Total AUC and Normalized Peak Intensity values calculated from the benchtop system datasets for all participants (P1–14). Values for participants 3–14 were extracted from the full 180 minutes of collected data. Due to early termination of data collection (explained above), parameters for participants 1 and 2 (indicated with an *) were extracted from 135 and 90 minutes of data respectively. On each graph, data is classified according to contrast agent dose (including the "*Corrected 200 mg*" data, where data collected at 200 mg was multiplied by a correction factor of 2.5 to allow comparison to data collected at 500 mg). Bars are color-coded based on the Fitzpatrick skin tone type for each participant (see legend). The numbers of participants in each Fitzpatrick group are shown in the legend. Dotted lines represent the values from the mean fluorescence vs. time curves (presented in Fig. 5.14) for each dose condition. **(A)** Peak Time parameter representing the time (in minutes) at which the signal reaches maximum intensity. **(B)** Total AUC. **(C)** Normalized Peak Intensity representing the maximum fluorescence intensity value for each dataset. 143

- 5.16 Total AUC values extracted from the mean normalized fluorescence intensity vs. time curves for both devices (*left* – compact sensor; *right* – benchtop system). Values were extracted from data shown in Fig. 5.12 (for compact sensor) and Fig. 5.14B (for Benchtop system). Statistically significant differences were observed between the 500 mg and 200 mg Total AUC values for both devices (identified by *). When a correction factor (of 2.5) was applied to the 200 mg Total AUC values ("*Corrected 200 mg*"; green bar), no statistically significant differences were observed when compared against the 500 mg data (for both devices), indicating an approximately linear relationship between dose and Total AUC. * – p value < 0.05; ns – not significant. 144
- 5.17 Characterization of compact sensor under different skin tone conditions. **(A)** Mean ($\pm$ STD) backscattered and fluorescence photodiode values from the first 10 minutes of the collected data (i.e., where no fluorescein signal is present) for each Fitzpatrick group (i.e. Type 1, 3 and 4/5). **(B)** Mean ($\pm$ STD) normalized intensity values extracted from the first 10 minutes of data for each Fitzpatrick group. **(C)** Mean ($\pm$ STD) total AUC values extracted from the full normalized fluorescence vs. time curves (180 minutes) for each Fitzpatrick group and each dose. Note that no error bar is shown for Type 4/5, 200 mg dose, as only one participant was categorized into this group. **(D)** Mean ($\pm$ STD) peak intensity values extracted from the full normalized fluorescence vs. time curves (180 minutes) for each Fitzpatrick group and each dose. Bars are color-coded according to Fitzpatrick skin tone type (see legends). The numbers of participants included in each group (including dose conditions) are shown in the legends (total number of participants: n=14). Dotted lines in A and B represent the mean values extracted from the curves averaged across all participants (i.e. from the curves presented in Fig. 5.12A for the normalized intensity values), where Fitzpatrick classification is not considered. Dotted lines in C and D are represent the values extracted from the mean curves presented in Fig. 5.12B 145

- 5.18 Characterization of compact sensor under different skin tone conditions – individual values. **(A)** Photographs of the hands and arms of three participants representing Fitzpatrick types 1, 3 and 5 (left to right respectively). **(B)** Individual backscattered (upper graph) and fluorescence (lower graph) photodiode values averaged over the first 10 minutes of data (i.e., where no fluorescein signal is present). **(C)** Individual normalized intensity values averaged over the first 10 minutes of data (i.e., where no fluorescein signal is present). Bars are color-coded according to Fitzpatrick skin tone type (see legends). The numbers of participants included in each group are shown in the legends (total number of participants: n=14). Fluorescein doses used for each participant are indicated on the graphs. Dotted lines represent the mean values extracted from the values averaged across all participants (i.e., with Fitzpatrick classification not considered). 147
- 5.19 Miniaturisation of the compact optical sensor. **(A)** Initial configuration of the compact sensor presented in the previous sections. Top and front view are shown. The top view also depicts the internal electronic configuration of the sensor, showcasing the differences in size between the electronics (integrated inside the metallic boxes) and the NI DAQ used to control and collect data. **(B)** First PCB design. Internal parts, including the amplification stages (denoted as “Amp stage”) and the LED driver, are identified. **(C)** Size comparison between the PCB designs and the LED and SMA connectors. **(D)** Final PCB design. As before, internal parts, including the amplification stages and the LED driver, are identified. **(E)** Current version of the miniaturisation developed in collaboration with Dr. Nilanjan Mandal. It integrates the final PCB design shown in (D). A battery source, as well as a touchable screen (WIO terminal) and an SD card port are included. As in the compact sensor shown in (A), the custom fibre probe is attached to the photodiodes and LED through SMAs connectors. The distal part of the probe is then attached to the participant using a 3D printed finger clip (not present in the image). 149

- 6.1 Compact fibre-optic fluorescence sensor used for transcutaneous assessment of intestinal permeability. **(A)** Schematic diagram of the compact fluorescence sensor; **(B)** Custom fibre probe diagram showing distal fibre arrangement (left) and full bundle (right). **(C)** Photograph of original compact sensor deployed in a clinical environment in the UK. The laptop shows the user interface developed for collection and pre-processing of data. The interface can be seen in more detail in Fig 5.6. A 3D printed finger clip is connected to the fibre tip. The fluorescein solution ready to be ingested by a volunteer is also shown in the photo. It contains 200mg of fluorescein mixed with 100ml of water. **(D)** Photograph of the replica compact sensor. A fibre-probe (similar to the original sensor in (C) and with the same configuration as in (B)) is connected to the sensor. The finger clip has been removed for a better visualization of the fibre-probe. This sensor is then connected to a laptop (as the sensor in (C)) to collect and process the fluorescence intensity data. **(E)** A close-up of the distal tip of the optical fibre probe used for both sensor (C) and (D). 153
- 6.2 Electronic configuration of Sensor 1 and Sensor 2. Two stage amplification circuit is implemented. The electronic configuration shared between the sensors is colored in black. Modifications applied specifically to Sensor 2 are shown in red. . 155
- 6.3 Calibration experiment setup. **(A)** (*top*) Experiment set up. Fibre probe (with the finger clip removed) is placed inside the 3D printed mount. The 3D printed mount prevents background light affecting the measurements. The fibre-probe tip is positioned perpendicular to the Fluorescence reflectance standard and the ND Filter. (*bottom*) Diagram of the set up. The different ND filters are placed in between the fibre-probe and the reflectance standard to modify the amount of light reflected to the fibre-probe. **(B)** (*top*) Photography of the LABSPHERE Certified Reflectance Standard used to assess the dynamic response of both compact sensors. (*middle*) Illustration of the different ND Filters. The background red line helps to visualize the different transparencies of the ND Filters. The lower the ND value, the higher the optical transmittance achieved. (*bottom*) Emission and Excitation spectra, according to the manufacturer, for the Reflectance standards shown in (B) (*top*). Excitation and Emission ranges for this reflectance standards are 300-360nm and 550.630nm, respectively, with an additional excitation band at 467nm. 156

- 6.4 Clinical setup of the compact transcutaneous fluorescence sensor (Sensor 2). **(A)** Clinical setup of the portable fluorescence sensor (main components indicated by annotations). The laptop contains the LabView interface developed to control and collect data from the sensor. **(B)** Photography of the room where Sensor 2 is deployed. Laptop and sensor are both easily accessible to the clinician/user. The participant is sat with the arm rested in a comfortable position and the 3D printed finger clip shown in (A) is attached to the forefinger of the participant before the experiment starts. **(C)** Photography of the St Augustine Clinic (Lusaka, Zambia) where Sensor 2 is deployed, and the Undernutrition study takes place. 158
- 6.5 *(left)* Image taken from Alshamy et al. [100] showing the morphometry measurements relevant for this chapter: VH - villus height (A), CD - crypt depth (C) and ESA - epithelial surface area (D). *(right)* Illustration of parameters VH and CD. In our case, the ESA parameter, represented in green in the left image (D), is also normalised by the size of the mucosa sample. 161
- 6.6 **(A)** Photodiode output signals from both of the sensors (Sensor 1 in blue and Sensor 2 in orange) for the different ND filters. Both photodiodes (Fluorescence photodiode in the left and Backscattered photodiode in the right) include a saturation region at a value of 5V, due to the configuration of the electronic circuit. Values used to calculate the Calibration Ratio are highlighted with a dotted red box, as they lie within the non-saturated and non-zero regions. These are the values from the 0.6, 0.8 and 1 ND filters. **(B)** Mean ratios and SD calculated from 10 repeats on 3 different days following equations 6.2, 6.3 and 6.4 for the output values from the 0.6, 0.8 and 1 ND Filters. *(left)* Individual ratios from each ND filter - 0.6 (left), 0.8 (middle) and 1.0 (right) - and Photodiodes, as well as the Normalised Intensity (Norm Int) values calculated following equation 3. The exact values can be observed in Table 6.4. *(right)* Final mean and SD Calibration ratios obtained for each photodiode as well as the Normalised Intensity are shown. These also correspond to the values presented in Table 3 - as the mean and SD values calculated across the 3 ND filters and the 3 days - and are used to correct for differences between the developed sensors (Sensor 1 and 2). 164
- 6.7 Photodiode and Normalised Intensity signals from the in vitro validation experiment. Different fluorescein concentration, ranging from 0 to 0.0017 mg/ml of fluorescein, were used to validate the Calibration factors presented in **Table 3** and Fig 6.6. Signals from Sensor 1 (blue), Sensor 2 (yellow) and corrected Sensor 2 (orange) are shown. **(A)** Output signals from the Fluorescence (left) and Backscattered (right) photodiodes. The saturation region is also included for the PD_F , as it represents the region above which the photodiodes are saturated, which in this case is 5V. **(B)** Normalised Intensity signal calculated using the values from the photodiodes. 166

- 6.8 Fluorescence Intensity signals collected from participants in Group 1 (Healthy volunteers) using the compact Sensor 1. **(A)** Time-resolved normalized fluorescence signals for participants 1-17. **(B)** Mean ($\pm$ STD) normalized fluorescence vs. time curves for participants in panel A. A wrong adjustment of the finger clip caused higher intensity values (from 0 to 20 minutes) to Participant UK8 (see panel A). This was resolved by adjusting the finger clip. However, this diffculted the extraction of baseline signals needed for a correct signal normalization. Therefore, Participant UK8 was not included for the calculation of the mean curve. **(C)** Total AUC values and Peak (maximum) intensity values extracted from the normalized intensity curves shown in panel A. Note that the mean values (both in Total AUC and Peak Intensity) are extracted from the averages observed in each curve in panel A. That is, the mean Peak Intensity shown do not correlate with the value observed on the curve shown in panel B due to variability in the time at which peak intensities were observed in each participant (see panel A). 168
- 6.9 **(A)** Linear regression model applied to Peak AUC vs L:M ratio (LMR) data. The goodness of fit of the linear model is represented by the R^2 value. Its corresponding p-value is also included. The equation fitted to the data (red line) is presented in the form of $y = mx + c$. **(B)** Linear regression model applied to the same dataset (Peak AUC vs LMR) but, this time, values from Participant 1 (UK1) are not considered. 172
- 6.10 Fluorescence Intensity signals collected from participants in Group 2 (potential undernourished volunteers) using the compact Sensor 2. **(A)** Time-resolved normalized fluorescence signals for participants 1-34. **(B)** Mean ($\pm$ STD) normalized fluorescence vs. time curves for participants in panel A. **(C)** Total AUC values and Peak (maximum) intensity values extracted from the normalized intensity curves shown in panel A. Note that the mean values (both in Total AUC and Peak Intensity) are extracted from the averages observed in each curve in panel A. That is, the mean Peak Intensity shown do not correlate with the value observed on the curve shown in panel B due to variability in the time at which peak intensities were observed in each participant (see panel A). All participants were classified as Fitzpatrick type 5/6. 174
- 6.11 **(A)** Linear regression model applied to Peak AUC vs L:R ratio (LRR) data. The goodness of fit of the linear model is represented by the R^2 value. Its corresponding p-value is also included. The equation fitted to the data (red line) is presented in the form of $y = mx + c$. **(B)** Linear regression model applied to the same dataset (Peak AUC vs LRR) but, this time, values from Participant 1 (UK1) are not considered. 177

- 6.12 Linear regression models applied to fluorescence parameter and morphometry data in Table 6.7 and 6.9. The goodness of fit of the linear model is represented by the R^2 value. Its corresponding p -value is also included. The equation fitted to the data (red line) is presented in the form of $y = mx + c$. **(A)** Linear regression model applied to Peak AUC vs villus height (VH) data presented in Table 6.7 and 6.9. Linear regression model applied to Peak Intensity vs ESA data presented in Table 6.7 and 6.9. **(C)** Linear regression model applied to epithelial surface area (ESA) vs villus height (VH) data. In this case, data includes values from within the entire cohort of participants recruited in Zambia ($n = 80$). Out of the 80 total participants recruited, only morphometry data from 60 participants was available. This group also includes the data from the 34 participants presented in Table 6.7 and 6.9. 181
- 6.13 Mean Photodiodes and Normalised signals extracted from the first 10 minutes of the datasets presented in Fig. 6.8 and 6.10. **(A)** Signals from compact sensor 1 for experimental group 1 with a total of 16 participants. Data from Participant UK8 was excluded from the analysis due to data collection problems (see Section 6.3.2 for more information). Data is classified in three Fitzpatrick types: type 1-2, type 3-4 and type 5-6 and color-coded accordingly. **(B)** Signals from compact sensor 2 for experimental group 2 with a total of 34 participants. Data is predominately classified as Fitzpatrick types 5-6 and color-coded accordingly. The total number of participants classified on each group is shown in the legend. 184
- 6.14 **(A)** Normalised fluorescence signals from each participant (*left*), as well as the mean and STD signals (*right*), for group 1 (blue) and group 2 (orange). The total number of participants in each group (group 1 $n = 16$ and group 2 $n = 34$) is also displayed. Fluorescence signals for each group were collected on different locations using the developed sensors displayed in Figure 6.1 for a total period of 180 minutes. **(B)** Scatter-box plots displaying the parameters extracted from the fluorescence vs time curves – Total AUC, Peak Intensity, Peak Time, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time. Box plots also show median values (red lines), 25th and 75th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for group 1 (UK - blue) and group 2 (Zambia - red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25th percentile or above the 75th percentile by more than 1.5 times the interquartile range. 186

6.15	Scatter-box plots of the Peak Intensity values from each participant in Group 1 – healthy and Group 2 – undernourished, categorised into three groups: sex (female – F or male – M), age (younger or older than 35 years old) and BMI (threshold of 25). (A) Peak Intensity values as a function of sex. (B) Peak Intensity values as a function of age, with the threshold established at 35 years old. (C) Peak Intensity values as a function of BMI, with a cut value at 25. * - p value < 0.05; ** - p value < 0.001.	189
6.16	Scatter-box plot displaying the individual and mean values of percentage of Lactulose (%L) extracted from group 1 (blue) and group 2 (red). Box plots also show median values (red lines), mean values (green lines), 25 th and 75 th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for group 1 (UK - blue) and group 2 (Zambia - red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25 th percentile or above the 75 th percentile by more than 1.5 times the interquartile range. Mean values are calculated using all data points, including the outliers.	191
6.17	Linear regression models applied to percentage Lactulose, Mannitol and Rhamnose presented in Table 6.5 and 6.14. The Pearson's correlation coefficient is represented by the ' r ' value. Its corresponding p -value is also included. (A) Correlations between the percentage of Lactulose recovery (%L) and the percentage of Mannitol recovery (%M), for group 1 (healthy controls), and the percentage of Rhamnose recovery (%R), for group 2 (undernourished). (B) Correlations between the percentage of Lactulose recovery (%L) and the Lactulose:Mannitol ratio (LMR), for group 1 (healthy controls), and the Lactulose:Rhamnose ratio (LRR), for group 2 (undernourished). In the case of group 2, data includes values from within the entire cohort of participants recruited in Zambia ($n = 80$), with a total of 42 data points. In the case of group 1, total number of data points is 13.	193
6.18	Phasor components extracted from the fluorescence signals from group 1 (blue) and group 2 (red). The Phasor plot, with the Real (g) and Imaginary (s) components of each participant, shows the distribution of the data points used for training and validation, as well as the testing data points (highlighted with a circle). Mean values for each group are displayed in filled circles (group 1 in blue and group 2 in orange). The graph also displays the decision boundary generated by the SVM algorithm. The confusion matrix generated from the testing of the SVM algorithm is also displayed. Metric values such as Accuracy, Specificity and Sensitivity are calculated from it.	196

7.1	Illustration of the paracellular and transcellular transport of molecules in a (A) Healthy and (B) Compromised epithelial barrier. The formation of a polarised cell monolayer is characterised by the presence of an apical compartment, in contact with the intestinal lumen, and a basolateral compartment, in contact with the circulatory system. The cells are linked together by the tight junction complexes. The transcellular pathway is an active transport mediated by transporters or endocytosis-exocytosis. The paracellular pathway, on the contrary, is a passive transport mediated by the pore or leak pathways.	202
7.2	<i>(top)</i> Schematic overview of a transwell cell culture insert. MDCK cells are seeded on top of the permeable membrane (pore size of 0.4 μm) of the transwell insert. The growth media is added to the transwell on both compartments. The compound, in this case, fluorescence molecules, are added to the apical compartment and the permeation to the basolateral compartment is measured using fluorescence spectroscopy. The arrows illustrate the direction of the transport of the molecules in the transwell containers. <i>(bottom)</i> Photography of the MDCK cells seeded on the transwell inserts, with the growth medium added to the chambers.	206
7.3	Microscope image of the MDCK cells at low <i>(left)</i> and high <i>(right)</i> confluence, prior to passaging.	206
7.4	TEER measurement to assess MDCK monolayer integrity. The TEER measurements uses two electrode, submerged separately in the apical and basolateral compartments, to measure the resistance across the membrane.	207
7.5	Process to calculate the different concentrations of EGTA. The stock concentration is used for the preparation of the maximum concentration of EGTA used in the protocol. The rest of the concentrations are prepared using a 1:2 titration. A similar process was followed to prepare the ethanol, while also preventing the ethanol from evaporating.	208
7.6	Configuration of the trasnwell inserts to assess the effect of different concentration of EGTA and Ethanol. The plates were label according to the image. A well was left as a control, with no cells seeded on the insert membrane. The same labels were used for the repeat in plate 2.	209
7.7	<i>(left)</i> Labelling of the plates used for the assessment of the permeation of the fluorescence dies - FITC-D: FITC-dextran; FL: Fluorescein; MB: Methylene Blue - at different concentrations (0% to 100%). <i>(right)</i> Dark plate used to measure the fluorescence intensity of the dyes, after the experiments. Apical and basolateral samples were added to the dark plate, to measure the fluorescence intensity remaining after 3 hours in both compartments.	212

7.8	(<i>left</i>) Labelling of the plates used for the assessment of the permeation of the fluorescence dyes - FITC-D: FITC-dextran; FL: Fluorescein; MB: Methylene Blue - at different concentrations (0% to 100%) and with different levels of disruption (Ethanol and EGTA at different concentrations). Each fluorescence dye had a 24-well plate with the different concentrations and disruptor conditions. (<i>right</i>) Control plate used to measure the permeability of the fluorescence dyes, without any disruption added to the wells.	214
7.9	TEER relative change calculated at 1.5 and 3 hours after the addition of the different concentrations of the disruptors. (<i>left</i>) Ethanol. (<i>right</i>) EGTA.	219
7.10	(A) TER measurements collected at the start of the experiment to assess the integrity of the monolayer barrier. These measurements were taken before the fluorescence dyes were added to the apical compartment. (B) Mean Fluorescence Intensity signals (from the two repeats) measured after 3 hours incubation for each fluorescence dye and concentration from the basolateral (<i>left</i>) and apical (<i>right</i>) compartment.	220
7.11	Mean Fluorescence Intensity signals measured after 3 hours incubation for each fluorescence dye and concentration from the basolateral (<i>left</i>) and apical (<i>right</i>) compartment. No cells nor disruptors were added to the medium.	222
7.12	(A) TEER relative change, calculated as $(T = 3\text{hours}) - (T = 0\text{hours})$, to assess the integrity of the monolayer barrier for the different disruptor conditions applied. Control values represent the change in TEER values for the wells with no disruptor applied. Different concentration of Ethanol (<i>left</i>) and EGTA (<i>right</i>) are colored coded depending on the concentration applied. (B) Mean Fluorescence Intensity signals (from the three repeats) measured, for each concentration of fluorescein, as well as the different disruptors applied: Ethanol (<i>left</i>) and EGTA (<i>right</i>). As before, the values are colored coded depending on the concentration of disruptor used in each case.	226
7.13	(A) TEER relative change, calculated as $(T = 3\text{hours}) - (T = 0\text{hours})$, to assess the integrity of the monolayer barrier for the different disruptor conditions applied. Control values represent the change in TEER values for the wells with no disruptor applied. Different concentration of Ethanol (<i>left</i>) and EGTA (<i>right</i>) are colored coded depending on the concentration applied. (B) Mean Fluorescence Intensity signals (from the three repeats) measured, for each concentration of FITC-dextran, as well as the different disruptors applied: Ethanol (<i>left</i>) and EGTA (<i>right</i>). As before, the values are colored coded depending on the concentration of disruptor used in each case.	228

7.14 **(A)** TEER relative change, calculated as (T = 3hours) – (T = 0 hours), to assess the integrity of the monolayer barrier for the different disruptor conditions applied. Control values represent the change in TEER values for the wells with no disruptor applied. Different concentration of Ethanol (*left*) and EGTA (*right*) are colored coded depending on the concentration applied. **(B)** Mean Fluorescence Intensity signals (from the three repeats) measured, for each concentration of Methylene Blue, as well as the different disruptors applied: Ethanol (*left*) and EGTA (*right*). As before, the values are colored coded depending on the concentration of disruptor used in each case. 230

List of Tables

4.1	Demographics of participants included in the study.	74
4.2	Parameters extracted from the fluorescence vs time curves for all 11 participants using Method 1.	83
4.3	Parameters extracted from the fluorescence vs time curves for all 11 participants using Method 2.	86
4.4	Parameters extracted from the fluorescence vs time curves using Method 3. . . .	87
4.5	Predicted parameters extracted from the single (1-term) exponential fitting curve.	90
4.6	Predicted parameters extracted from the double (2-term) exponential fitting curve.	93
4.7	Phasor components:	98
4.8	Statistical analysis comparing the datasets using the parameters extracted through each method.	102
4.9	Confusion matrix extracted from the SVM algorithm.	108
5.1	Demographics of the study participants and the respective ingested dose of fluorescent dye.	128
5.2	Pearson's correlation coefficients and p-values	137
5.3	Parameters extracted from the datasets presented in Fig. 5.10 and Fig. 5.11 . . .	138
5.4	Statistical Analysis of both systems	139
6.1	Demographics of the study participants in Group 1	159
6.2	Demographics of the study participants in Group 2	160
6.4	Calibration output voltages from Sensor 1 and 2 using the ND filters.	165
6.5	Parameters extracted from Group 1 using Sensor 1.	169
6.6	Pearson's correlation coefficients (r) and p-values	171
6.7	Parameters extracted from Group 2 using Sensor 2.	175
6.8	Pearson's correlation coefficients (r) and p-values	176
6.9	Morphometry parameters extracted from the duodenal biopsies from Group 2. . .	179
6.10	Pearson's correlation coefficients (r) and p-values	180
6.11	Pearson's correlation coefficients (r) and p-values	182
6.14	Percentage of Lactulose (%L) and Rhamnose (%R) recovery from participants in group 2	190
7.2	Ethanol and EGTA solutions	209
7.3	Fluorescence concentrations	212

7.4	Assessment of the permeation of the fluorescence dyes under normal (no disruption) conditions.	221
7.5	Statistical analysis	223
7.6	Assessment of the permeation of the fluorescence dyes after 3 hours (no cells) .	224
7.7	Percentage of <i>FLUORESCCEIN</i> fluorescence intensity,	227
7.8	Percentage of <i>FITC-Dextran</i> fluorescence intensity,	229
7.9	Percentage of <i>Methylene Blue</i> fluorescence intensity,	229

Acronyms

AUC Area Under the Curve.

BMI Body Mass Index.

CD Crypt Depth.

CEM Confocal Endomicroscopy.

DMEM Dulbecco's Modified Eagle Medium.

DRS Diffuse Reflectance Spectroscopy.

EE Environmental Enteropathy.

EGTA Ethylene glycol tetraacetic acid.

ESA Epithelium Surface Area.

FAD Flavine Adenine Dinucleotide.

FITC Fluorescein isothiocyanate.

FOT Fractional Optical Transmittance.

FWHM Full-width half-maximum.

GCP Good Clinical Practice.

GI Gastrointestinal.

IBD Inflammatory bowel disease.

IBS Inflammatory bowel syndrome.

L:M Lactulose:Mannitol.

L:R Lactulose:Rhamnose.

LED Light-emitting Diode.

LOOCV Leave-one-out cross-validation.

LRR Lactulose:Rhamnose Ratio.

MB Methylene Blue.

MDCK Madin-Darby Canine Kidney.

NADH Nicotinamide Adenine Dinucleotide.

ND Neutral Density.

NI National Instruments.

PEM Protein-Energy Malnutrition.

PET Polyethylene Terephthalate.

SMA SubMiniature A.

SVM Support vector machine.

TEER Transepithelial Electrical Resistance.

TGFR Transglomerular filtration rate.

TJ Tight Junction.

VCR Villus-to-Crypt Ratio.

VH Villus Height.

WHO World Health Organisation.

Chapter 1

Thesis Overview

The outline of the thesis is as follows:

Chapter 2 introduces the topic of gastrointestinal permeability, including the physiology of the gut barrier and the pathologies associated with an increased gut permeability or “leaky gut”, focusing on Environmental Enteropathy (EE) and undernutrition. The chapter also reviews the current clinical techniques to assess and monitor changes in gut permeability and define the limitations associated with these techniques. We finish the chapter by highlighting the need for better, non-invasive and more robust tools for the assessment of gut barrier function.

Chapter 3 then explores the use of optical approaches for the assessment of gut permeability, including the use of transcutaneous spectroscopy in combination with fluorescence contrast agents. This chapter covers the topic of spectroscopy focusing on fluorescence spectroscopy and the use of endogenous and exogenous fluorophores on clinical diagnostics. In addition, the state of the art of fluorescence spectroscopy for the assessment of gut permeability is covered before introducing the use of transcutaneous fluorescence spectroscopy as a potential tool for the non-invasive assessment of gut permeability.

Recognising the limitations of the current clinical techniques to assess gut permeability and the potential of transcutaneous fluorescence spectroscopy, **Chapter 4** introduces the technology developed by Dr Thompson and the work done to further validate the approach. First, we present a study in healthy volunteers with different induced gut permeability conditions and the collection of data using the fluorescence spectroscopy system. In this chapter, we explore the potential of this spectroscopy system to differentiate between the different stages of gut permeability, focusing on the development of processing and analysis strategies to capture relevant information from the data collected. This chapter, therefore, focuses on investigating multiple computational techniques for feature extraction and data classification. The results from this chapter are used to understand the effect of these approaches in the classification and interpretation of the data and their applicability in the following chapters.

The limitations associated with the device introduced in **Chapter 4** are further investigated in **Chapter 5**, with the focus on improving areas such as deployability and cost-effectiveness. This chapter

then presents the development of a compact transcutaneous fluorescence sensor for the assessment of gut permeability to be applied in further experimental work. The chapter covers the optical, electronic and software design and development of the device, together with the validation approaches in vitro and in vivo carried out for the evaluation of its performance. To further validate the device, a clinical study in healthy volunteers investigated the performance of the sensor under different fluorophores concentrations and skin tones. Lastly, the potential of the device for assessment of gut permeability and its further miniaturisation are discussed.

Given the results presented in **Chapter 5**, **Chapter 6** focuses on the deployment of the device in an undernutrition clinical study in a low-income setting. In this case, the fluorescence permeability profiles of two cohorts - healthy controls from the UK and undernourished volunteers from Lusaka (Zambia) - are investigated using the portable device. To collect data from both groups simultaneously, the development of a replica of the device from **Chapter 5** is presented. Upon calibration of both devices, fluorescence signals collected from both cohorts are analysed by implementing some of the strategies presented in **Chapter 4**. To validate the compact sensors even further, standard measures of gut barrier integrity, such as dual sugar permeability assays or duodenal biopsies, are correlated with the fluorescence signals.

Finally, **Chapter 7** investigates the permeation of fluorescein and other fluorescence contrast agents in 2D monolayer models. The primary objective is to understand the fluorescein permeation observed in **Chapter 5 and 6** through a controlled in vitro model. This includes the use of malicious components to induce various stages of barrier dysfunction. In addition, other alternative fluorophores widely used in the clinical and in vitro models are investigated with the objective of further improve the assessment of gut permeability.

The thesis is concluded in **Chapter 8** with a summary of the findings from each chapter, including the limitations associated with the studies and techniques described on each section. Potential future directions of the work here presented are also discussed with the focus on the development of improved tools to provide further understanding on the mechanisms and interactions behind gut permeability.

1.1 Publications

The work presented in this thesis has resulted in the following publications and conferences.

Journal articles:

- J. Gan, **E. Monfort Sánchez**, J. Avery, O. Barbouti, J. Hoare, H. Ashrafian, A. Darzi, A.J. Thompson. "Non-invasive assessment of intestinal permeability in healthy volunteers using transcutaneous fluorescence spectroscopy", *Methods and Applications of Fluorescence*, Vol. 10, 044014, [dx.doi.org/10.1088/2050-6120/ac9513](https://doi.org/10.1088/2050-6120/ac9513)
- **E. Monfort Sanchez**, J. Avery, J. Gan, J. Qian, N. Mandal, A. Agarwal, M. Mwiinga, R. Banda, A. Darzi, P. Kelly, A. J. Thompson, "Transcutaneous fluorescence spectroscopy: development and characterization of a compact, portable, and fiber-optic sensor," *Journal of Biomedical Optics*, 29(2) 027003, 2024, doi.org/10.1117/1.JBO.29.2.027003

Conference papers:

- **E. Monfort Sánchez**, J. Avery, A. Darzi, and A. J. Thompson, "Development of a compact fluorescence spectroscopy sensor for non-invasive monitoring gut function," in *Biophotonics Conference: Biomedical Optics 2022 (Translational, Microscopy, OCT, OTS, BRAIN)*, Technical Digest Series (Optica Publishing Group), 2022, paper TS2B.6, doi.org/10.1364
- J. Gan, **E. Monfort Sánchez**, H. Ashrafian, A. Thompson, A. Darzi, "Tu1655: use of a non-invasive, portable, fibre-optic fluorescence spectrometer to measure gut permeability- is it feasible?" in *Digestive Disease week 2022 conference, Gastroenterology*, Volume 162, Issue 7, Supplement, 2022, Page S-1024, ISSN 0016-5085, [doi.org/10.1016/S0016-5085\(22\)62437-5](https://doi.org/10.1016/S0016-5085(22)62437-5)
- **E. Monfort Sánchez**, J. Avery, A. Thompson, "Development of a wearable fluorescence sensor for non-invasive monitoring gut permeability", in *The Hamlyn Symposium on Medical Robotics: MedTech Reimagined conference*, 2022, dx.doi.org/10.31256/HSMR2022.32
- T. Fletcher, **E. Monfort-Sanchez**, M. Keshavarz, J. Avery, H. Ashrafian, A. Darzi, A. J. Thompson, "Quantifying hypoxia with diffuse reflectance spectroscopy for advanced prognostication and real-time response monitoring in rectal cancer: an in vivo feasibility study," *Proceedings SPIE 12372, Optical Fibers and Sensors for Medical Diagnostics, Treatment and Environmental Applications XXIII*, 1237206, San Francisco, 2023, doi.org/10.1117/12.2648827
- **E. Monfort Sánchez**, J. Avery, J. Gan, J. Qian, M. Mwiinga, R. Banda, J. Hoare, H. Ashrafian, A. Darzi, P. Kelly, A. Thompson, "A compact fluorescence sensor for low-cost non-invasive monitoring of gut permeability in undernutrition," *Proceedings SPIE 12369, Optics and Biophotonics in Low-Resource Settings IX*, 1236904, San Francisco 2023, doi.org/10.1117/12.2649030
- N. Mandal, **E. Monfort Sánchez**, J. Avery, J. Gan, J. Qian, M. Mwiinga, R. Banda, J. Hoare, H. Ashrafian, A. Darzi, P. Kelly, A. Thompson, "A wireless semi-wearable sensor for assessment

of gut function in low-resource setting” Proceedings SPIE 12832-8, Optics and Biophotonics in Low-Resource Settings XI, Wearable and related technologies, San Francisco 2024, [12832-8](#)

Chapter 2

Clinical Motivation

2.1 Gastrointestinal Permeability

The gastrointestinal (GI) barrier plays a crucial role in maintaining homeostasis by regulating the transport of molecules between the intestinal lumen and the circulatory system. This process, known as molecular transportation, involves intricate mechanisms that ensure selective permeability while preventing the entry of detrimental substances, such as toxins, pathogens and allergens [1, 2]. Understanding the dynamics of molecular transportation across the GI barrier is essential for elucidating the pathophysiology of various GI disorders and developing improved diagnostic tools.

In this section, we provide an overview of the processes involved in molecular transportation across the GI barrier, with a focus on the paracellular pathway and the consequences associated with the integrity of the epithelial barrier. In addition, we will also discuss about the limitations of the current approaches for assessing gut permeability and the need for improved technologies to further understand the complexity of this process.

2.1.1 Gastrointestinal Epithelium: a multifunctional barrier

The gastrointestinal epithelium serves as a critical barrier between the external environment of the gut lumen and the underlying tissues [1–3]. Its structure varies along the intestinal tract, with distinctive features along its different regions [4, 5]. Fig. 2.1 illustrates the different regions within the small intestine.

In the small intestine, for example, the epithelial surface is characterised by finger-like projection called villi, which extend into the lumen [6–8]. These villi, and the microvillus within it, significantly increase the surface area to maximise the absorption of nutrients [4, 8]. At the base of these villi, there are invaginations known as crypts of Lieberkühn [4, 8]. These structures contain stem cells responsible for the continuous renewal of the epithelial cells, which occurs generally every 4–5 days [9]. Within the gastrointestinal barrier, multiple layers contribute to its function. The outermost layer, the mucosal layer, provides lubrication and protection against luminal content, such as pathogens associated molecular peptides [1–3]. Beneath the mucosal layer lies the epithelial layer. It is composed of various cell types responsible for nutrient absorption (enterocytes), barrier protection (goblet and Paneth cells) and hormone and enzymes segregation (enteroendocrine, neuroendocrine and Paneth cells) [1–3, 8]. The harmonised function of all these cells helps maintain gut homeostasis and protect against pathogens, ultimately ensuring the efficient absorption of nutrients [1, 2]. In addition, specialised immune cells, such as intraepithelial lymphocytes and lamina propria immune cells, are also present within the epithelial layer, contributing to immune surveillance/regulation and defence against pathogens [1, 2].

Overall, the structure of the gastrointestinal epithelium plays an important role in multiple physiological processes, essentially maintaining a correct function of the gastrointestinal tract to ensure

the overall health of the individual [1, 2]. In this chapter, we will focus on the translocation of luminal content from the small intestine to the systemic circulation and the multiple factors contributing to the integrity of the GI epithelial barrier.

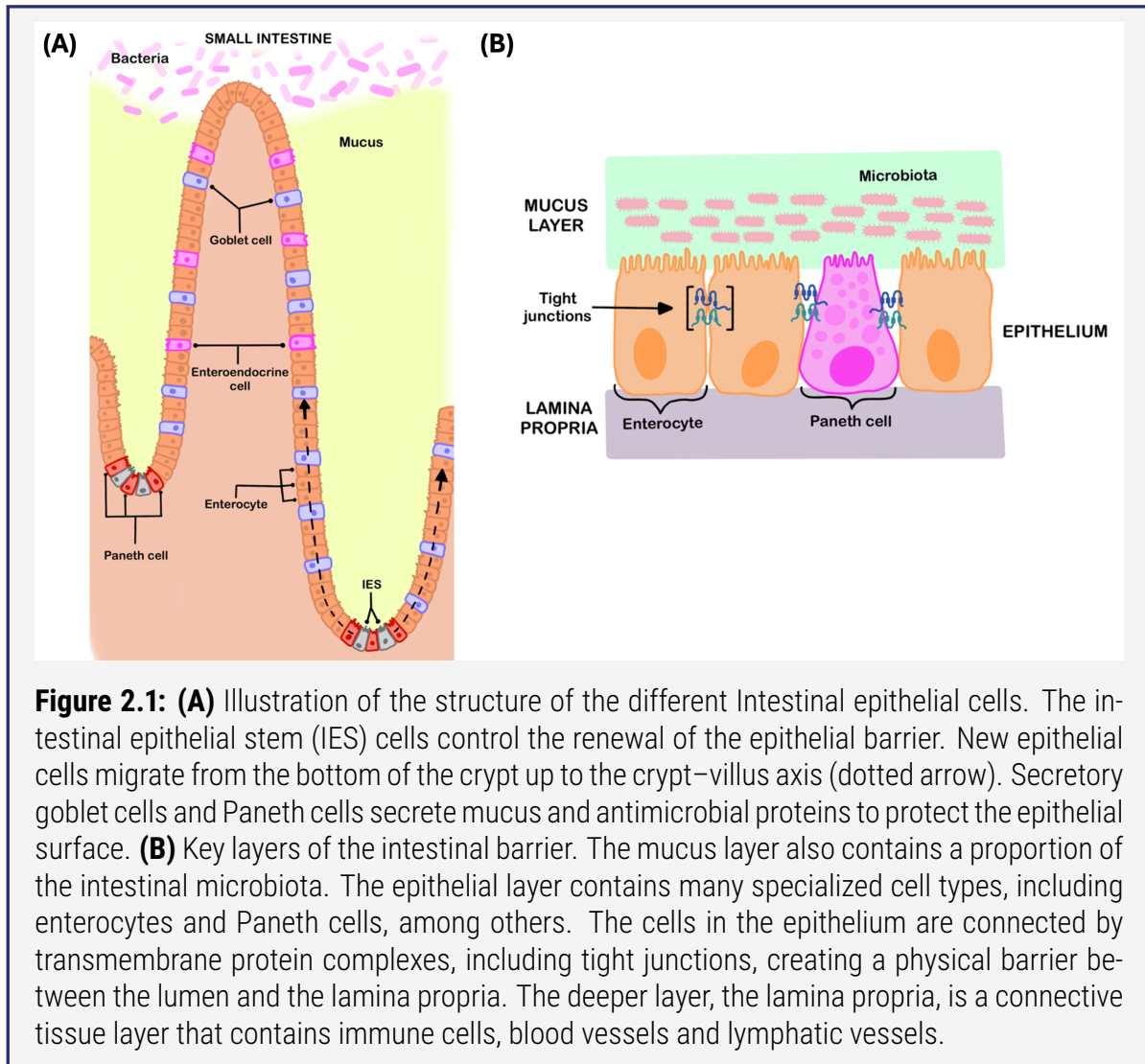
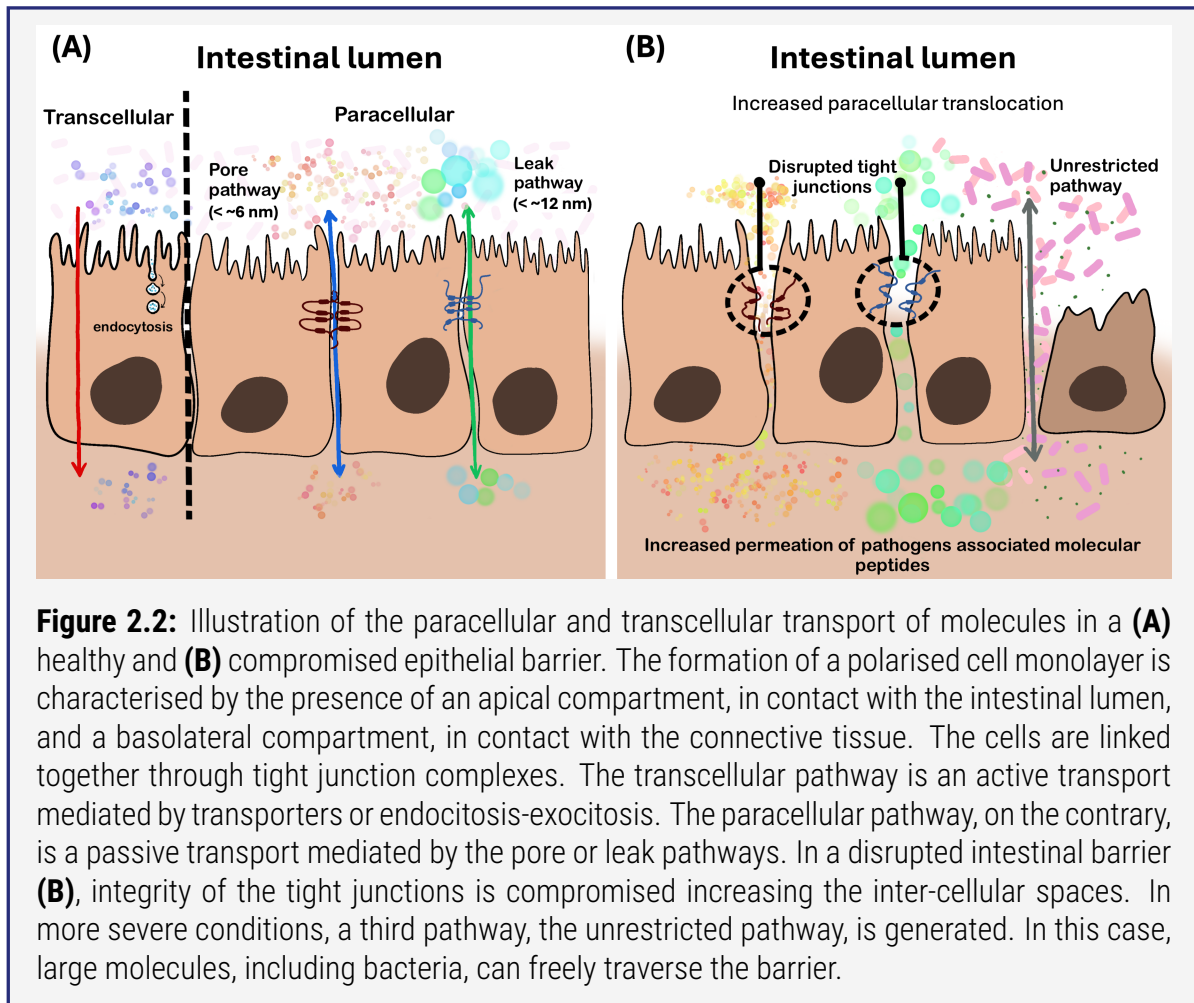


Figure 2.1: (A) Illustration of the structure of the different Intestinal epithelial cells. The intestinal epithelial stem (IES) cells control the renewal of the epithelial barrier. New epithelial cells migrate from the bottom of the crypt up to the crypt–villus axis (dotted arrow). Secretory goblet cells and Paneth cells secrete mucus and antimicrobial proteins to protect the epithelial surface. **(B)** Key layers of the intestinal barrier. The mucus layer also contains a proportion of the intestinal microbiota. The epithelial layer contains many specialized cell types, including enterocytes and Paneth cells, among others. The cells in the epithelium are connected by transmembrane protein complexes, including tight junctions, creating a physical barrier between the lumen and the lamina propria. The deeper layer, the lamina propria, is a connective tissue layer that contains immune cells, blood vessels and lymphatic vessels.

2.1.2 Molecular Transportation across the Gastrointestinal Barrier

The translocation of molecules from the GI lumen to the systemic circulation is highly mediated by the transcellular and paracellular pathways [10–13]. Fig 2.2 illustrates the different transportation mechanisms present across GI epithelium.

The **transcellular pathway** involves the transport of molecules and ions across the epithelium of the gut through the plasma membrane of the epithelial cells [3, 14]. The transcellular transport of molecules is dependent on the properties of the molecule to be transported, such as size, charge and lipophilicity [13],[15]. Their translocation from the lumen to the interspace can occur through various mechanisms, including passive diffusion, facilitated diffusion, active transport, and endocytosis [13, 15]. Passive diffusion is driven by concentration gradients and occurs for small,



lipophilic molecules that can freely diffuse across the lipid bilayer of epithelial cells [13]. Facilitated diffusion involves the use of carrier proteins, such as peptide and amino acid transporters, to transport molecules across the epithelial membrane, typically down their concentration gradient [15]. Active transport, on the other hand, requires energy expenditure and is often employed to transport ions and nutrients such as peptides, against their concentration gradient [13]. Lastly, endocytosis and transcytosis refer to the vesicle-mediated process by which cells internalize macromolecules and particles by invaginating a portion of the cell membrane to form vesicles that are subsequently transported across the cell and released into the intracellular space or vice versa [3],[16].

Alongside transcellular pathway, molecules, such as ions, metabolites and macromolecules can also traverse the GI barrier via the **paracellular pathway** [3, 14, 17], see Fig. 2.2. This route refers to the movement of molecules between epithelial cells via the intercellular spaces, primarily mediated by tight junctions (TJs) [18, 19]. These tight junctions are complex structures that play an important role on regulating the translocation of molecules through the paracellular pathway and on maintaining the functionality and polarity of the GI epithelial barrier [18–21]. They consist of a tight network of transmembrane proteins, such as claudins, occludins and zonula occludens, that

seal the intercellular gaps between adjacent epithelial cells, forming a selective barrier that regulates paracellular permeability [11, 18, 19, 22]. The paracellular translocation of molecules through the TJs typically involves two distinct routes: the leaky and pore pathways. The leak pathway functions as a route with limited capacity and transportation specificity primarily determined by molecular size rather than charge. As studied in cellular and animal models, it has been estimated that the leak pathway allows passage of molecules with a diameter of up to ≈ 12 nm through the gut wall barrier [14, 17, 23]. The pore pathway, on the contrary, serves as a high-capacity route that exhibits both molecular size and charge selectivity. In this case, it was estimated that molecules with diameter up to ≈ 0.6 nm can traverse the gut wall barrier using this pathway [14, 17, 23].

The composition and integrity of tight junctions are dynamically regulated by various factors, including cytokines, immune signals, dietary components, and microbial metabolites [11, 24]. Disruption of the epithelial barrier due to tight junction integrity and/or cell viability can lead to increased paracellular permeability, generating an unrestricted pathway for molecules to translocate from the GI tract to the systemic circulation [15]. In such cases, the paracellular flux of large and potentially pathogenic molecules such as antigens, toxins, and pathogens into the bloodstream contributes to the pathogenesis of various disease conditions both intestinal and systemic [25, 26],[27, 28],[29]. Consequently, the process of molecular transportation across the GI barrier needs to be tightly regulated to maintain homeostasis and prevent the translocation of pathogenic substances into the circulatory system.

2.1.3 Gastrointestinal Pathologies - the role of gut permeability

As we have previously mentioned, the integrity of the gastrointestinal (GI) epithelium is essential for maintaining the barrier function of the gut, significantly contributing to the overall health of the body. However, various factors contribute to the impairment of the GI epithelial integrity, leading to disruptions in barrier function and increased permeability.

The gut lumen is a non-sterile space, which contains a significantly dense and diverse microbiome [1, 30]. In normal conditions, when the integrity of the GI barrier is not compromised, the epithelial monolayer acts as a selective physical barrier, separating the microbiota from the circulatory system and maintaining the physiological balance of both the lumen and the circulatory system content [26, 30, 31].

Microbiota imbalance, or dysbiosis occurs when there is an alteration in the composition or diversity of the microbial community inhabiting the GI tract. This imbalance can result from antibiotic use, dietary changes, environmental pollutants or toxins and/or any underlying medical conditions that compromise the delicate balance of microbial populations [2]. An increase in gut permeability, together with the translocation of bacterial products, can trigger immune and inflammatory responses that can lead to more severe conditions [25, 32, 33]. In patients with inflammatory bowel disease (IBD), for example, some proinflammatory cytokines can play a significant role in

the pathogenesis of the disease by directly damaging the gut barrier, exacerbating the condition [33, 34]. Other factors, such as physiological stress and genetic predisposition, also play an important role in shaping the gut barrier integrity [33, 35].

It is known that all the factors mentioned above often coexist in a detrimental cycle, exacerbating any underlying health condition, both systemic and intestinal [36]. Although there is limited knowledge about the mechanisms underlying the relationship between increased gut permeability and disease, the disruption of gut barrier and the resulting increase of gut permeability, poses immense health challenges [11]. It is therefore why the integrity of the intestinal barrier is gaining recognition as a significant contributor to various pathophysiological conditions, including inflammatory bowel disease (IBD), coeliac disease, Environmental Enteropathy (EE) and malnutrition.

The association between intestinal barrier dysfunction and intestinal disease has been widely reported in IBD patients [12]. Consistent with this idea, increased levels of intestinal permeability have also been found on Crohn's disease patients prior to relapse [37–39]. In patients with coeliac disease, compromised intestinal barrier function has also been reported, where the disease activity, and thus, the immune response to the presence of gluten/gliadin, has been positively correlated with the increase of gut barrier damage and permeability [40, 41]. In addition, structural changes of the epithelial barrier, specifically a reduction in surface area and reactive crypt hyperplasia, have been reported in patients with coeliac disease [25]. However, the relationship between gut permeability and gluten is not clearly understood [42, 43].

Environmental factors, such as urbanization/pollution, antibiotic use, hygiene status and microbial exposure, have an important influence on the distribution of diseases around the world [44–47]. For example, the incidence and prevalence of conditions such as IBD or coeliac disease has been predominantly reported in regions such as North America and north-western Europe [48–50]. Environmental influence of gluten-containing, ultra-processed or proinflammatory food, predominant in developed countries, could be an explanation to this distribution [47, 49]. In addition, other pathological conditions, such as acute diarrheal and Environmental Enteropathy (EE), have been predominantly reported in developing regions, such as South America, Africa and South Asia, with poor sanitation, contaminated water and increased exposure to infectious agents [51–54].

EE, first known as “tropical enteropathy” [55], is a poorly understood condition that manifests as complex structural and functional changes in the small intestine with reduced intestinal surface area, chronic inflammation, increased intestinal permeability (or leakiness) and reduced absorption of nutrients [54, 56, 57].

2.1.3.1 Undernutrition

Malabsorption of nutrients is often driven/associated with a compromised GI barrier integrity. As we have seen, abnormal increased permeability, commonly referred to as “leaky gut syndrome”, allows pathogens, toxins, and undigested food particles to enter the bloodstream, triggering sys-

temic inflammation and immune responses. Immune dysregulation may increase susceptibility to infections, allergies, and autoimmune diseases. These conditions are also accompanied with structural changes in the GI epithelium, including thinning of the mucosal layer and alterations in tight junction proteins, which significantly impact the efficient absorption of nutrients [18, 53, 55, 58]. Disruption of the gut barrier and the abnormal absorption of nutrients can further exacerbate nutritional deficiencies and impair overall health [54, 59, 60].

Malnutrition, as defined by the World Health Organisation (WHO), is a condition that arises when there is an excessive or deficient intake of essential nutrients, accompanied with nutrient imbalance or impaired nutrient utilization [61]. It can manifest in various forms, ranging from undernutrition to overweight and obesity, and it affects not only individuals, but also their families. Undernutrition stands as a global health concern with multifaceted implications. The latest report from WHO showed that undernutrition is associated with approximately 45% of deaths among children under the age of 5 [61], mainly located in low- and middle-income countries. It is a condition marked by an inadequate intake of essential nutrients crucial for maintaining health and well-being [62–64]. As established by the WHO, undernutrition compromises five subgroups: underweight, wasting, stunting and deficiencies in vitamins and minerals. A calorie undernutrition, where individuals fail to meet their daily energy requirements, results in weight loss, diminished energy levels and a pervasive sense of weakness [65, 66]. Protein-Energy Malnutrition (PEM) is a severe form of undernutrition that manifests as a both calories and protein intake deficiency, often causing growth difficulties (stunting), muscle wasting, edema and heightened susceptibility to infections [63, 67]. Micronutrient-related malnutrition, such as essential vitamins and minerals deficit, presents an important threat to the health and development of individuals [68–70].

The prevalence and incidence of undernutrition have not been improving significantly all around the world despite the global efforts to combat hunger and malnutrition [71–74]. It remains a critical issue in certain regions, especially in sub-Saharan Africa and South Asia [61, 72, 74–76]. Over the past year, continuous effort has been focused on developing strategies to improve the nutrient absorption of patients affected by undernutrition [60, 77–79].

The evidence showed that the effectiveness of complementary feeding in undernourished children is relatively thin [80–84]. This could be tentatively attributed to the variability between studies, populations and strategies implemented [85]. In addition, the malabsorption of nutrients might not be fully resolved with complementary feeding due to underlying problems related to gut dysfunction [84, 85]. Multiple studies have shown bidirectional relationship between gut permeability and undernutrition, with alterations in barrier function contributing to malnutrition and vice versa [58]. However, the mechanisms and interactions behind changes in gut permeability and pathologies, such as undernutrition, are still not well understood. In addition, available clinical information is often correlative, making it difficult to separate cause from effect [86, 87]. In the following section, we discuss the current techniques used in the clinic to assess changes in gut permeability.

2.2 Current diagnostic landscape for the assessment of gut permeability

The current diagnostic landscape of clinical approaches for the assessment of gastrointestinal (GI) permeability encompasses a diverse group of methods. These approaches aim to evaluate the integrity of the gut barrier and identify abnormalities that may contribute to disease pathogenesis, as we have previously discussed. Traditional methods include dual sugar permeability tests, such as the Lactulose:Mannitol test, to provide information about the GI paracellular pathway (and in extension tight junctions), assessment of plasma, fecal and urine biomarkers, and endoscopic biopsies that provide a morphological description of the gut epithelium [3, 15, 88]. Additionally, emerging technologies, such as spectroscopy or confocal laser endomicroscopy, offer more advance alternatives for assessing gut permeability [89–92].

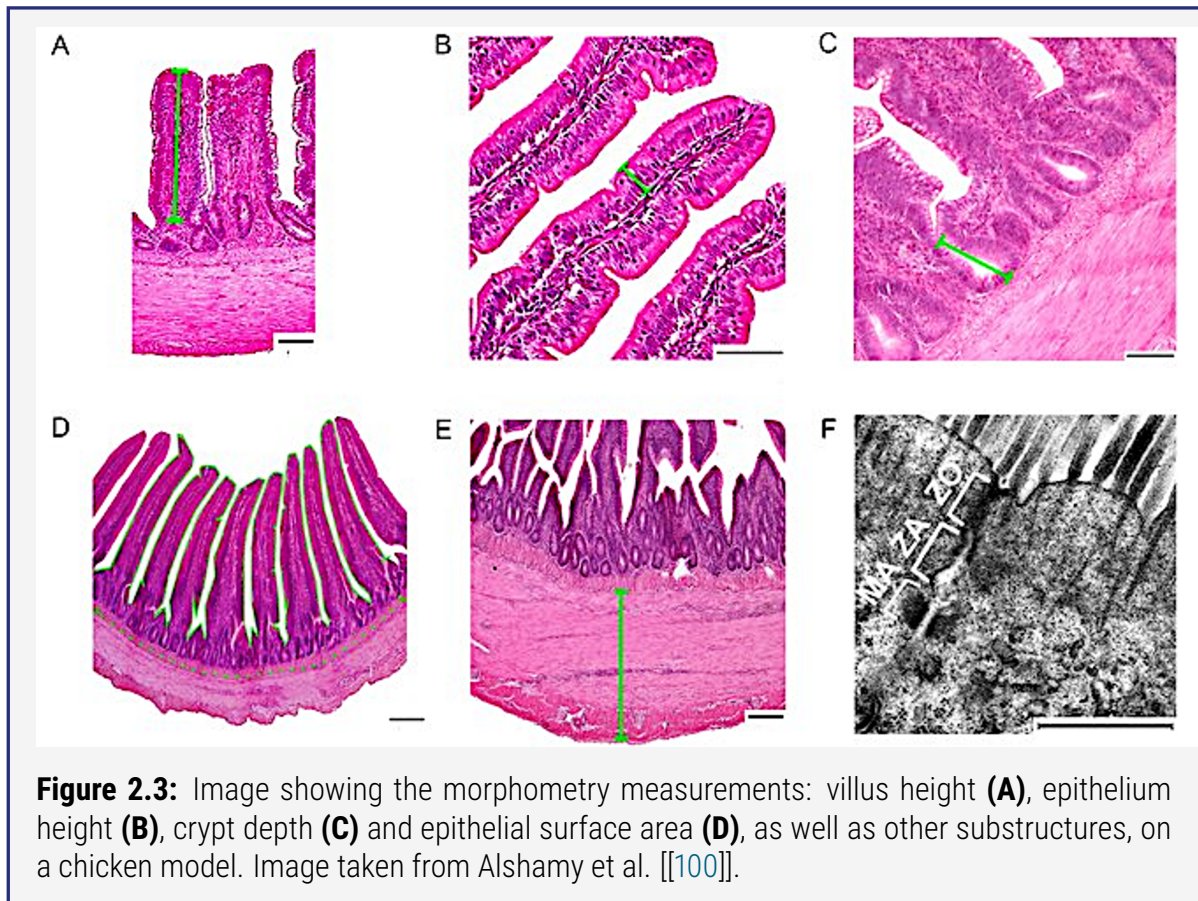
In the following sections, we will delve deeper in some of these approaches to understand the current landscape within the clinic for the assessment of changes in gut permeability and their current limitations.

2.2.1 Endoscopic biopsies

Endoscopic biopsies are one of the gold standards for assessing morphology changes of gut barrier in undernutrition, but it is also widely used for diagnosing other GI conditions such as IBD or coeliac disease [93–97].

By definition, endoscopic biopsies encompass the collection of tissues samples from both upper and lower parts of the gastrointestinal tract during an endoscopic procedure (including colonoscopy). These samples are processed in a laboratory facility, where they undergo histological staining and microscopic examination. The evaluation of the integrity of the gut barrier focuses on analysing various aspects of the sample, including the mucosal architecture, presence of inflammation, as well as abnormal structural changes [96, 98]. Biopsy markers, such as villus height (VH), crypt depth (CD), villus to crypt ratio (VCR) and epithelial surface area (ESA) are commonly used for the evaluation of the structural integrity of the gut [98, 99]. Fig. 2.3 illustrated some of these features.

In undernourished individuals, endoscopic biopsies collected from the upper part of the small intestine often reveal morphological changes that are indicative of impaired gut barrier. Some of these changes, for example, include villous atrophy and crypt hyperplasia [96]. Villus atrophy is characterised by the shortening or flattening of the villi, reducing the absorptive surface. Crypts hyperplasia, on the contrary, may occur as a compensatory response to damage or loss of the villi. In this case, an increase in the number and depth of the intestinal crypts will occur as a measure to renew the epithelial cells and maintain intestinal integrity [101][102]. More importantly, duodenal



endoscopic biopsies collected from undernourished children also revealed reduced epithelium surface area (ESA), lamina propria inflammation and decreased goblet cell density [96, 98, 99, 103]. All these morphological changes are clearly impacting the functionality of the gut epithelium, exacerbating the pathogenesis of the condition [104]. In the case of children, these morphology changes can critically affect their future development, especially for children under the age of 5 years old [105, 106].

Although endoscopic biopsies are valuable tools for diagnosing gastrointestinal diseases and assessing gut epithelium integrity, the procedure is invasive and may cause discomfort or complications, including bleeding and perforations [107–109]. This is especially important in undernourished individuals with already in a weakened, inflammatory state and prone to infections. For children, in particular, because of the invasiveness of the procedure, several aspects need to be considered during an endoscopy, including any possible complication [110–112]. In addition, obtaining informed consent from parents or guardians may be challenging, especially in low- and middle-income populations with limited resources and cultural differences [110–112].

Overall, while endoscopic biopsies are highly invasive, they provide valuable information about the morphology of the GI tract, and the structural changes involving conditions affecting the gastrointestinal tract. However, the samples only represent a small region of the gastrointestinal tract and do not provide any functional information. In addition, confounding factors, such as inflammation,

infections or concurrent pathologies (i.e., HIV), can potentially complicate the interpretation of the results.

2.2.1.1 Confocal endomicroscopy

Other approaches, such as confocal endoscopic microscopy (CEM), allows for the *in vivo* visualisation of the gastrointestinal mucosa, and their characteristic cellular and subcellular structures, during rutinary endoscopies [113–115]. The primary advantage of CEM is the ability to provide real-time, high-resolution images of the surface of the intestinal tract, without the need for tissue excision. Unlike endoscopic biopsies, CEM also allows for rapid assessment of GI barrier function by evaluating the permeation of a fluorescence contrast agent, such as fluorescein through the epithelium, which can be particularly useful for identifying areas with early signs of gut barrier dysfunction [116]. Compared to endoscopic biopsies, CEM does not required tissue sampling or histological analysis. However, interpretation of the CEM images requires specialised expertise, and the technology relies on an expensive equipment and the use of an endoscope, which does not remove the invasiveness of the procedure and associated complications [113, 115]. In the next section we explore some less invasive approaches to assess gut permeability such as the use of dual sugar permeability tests.

2.2.2 Permeability tests

The dual sugar permeability test is a diagnostic tool widely used to assess gastrointestinal permeability, specifically the integrity of the intestinal epithelial barrier in conditions such as malnutrition [117], celiac disease [118], Crohn's disease [119], active inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [120], among others. These tests, entail the oral administration of inert/non-metabolized, water-soluble molecules that are absorbed through the intestinal mucosa into the bloodstream and are subsequently recovered in the urine. Because of their methodology, these approaches focus on evaluating the paracellular permeability of the ingested sugar molecules, providing a measure of Tight Junction integrity.

2.2.2.1 Lactulose:Mannitol permeability test

The Lactulose:Mannitol (L:M) permeability test has been used widely for *in vivo* assessment of intestinal barrier dysfunction. Lactulose, a synthetic disaccharide, was originally developed as a laxative in the 1960s [121, 122]. Its poor intestinal absorption in undistorted intestinal epithelium due to its large molecular weight (342.30 Da) made it an attractive candidate for assessing mucosal integrity [123, 124]. Mannitol, on the contrary, is a naturally occurring monosaccharide that permeates more easily through small channels, such as the water pores in the intestinal membrane, due to its smaller molecular weight (182.17 Da), therefore serving as marker of normal intestinal absorption [125].

The earliest application of the lactulose-mannitol permeability test focused on understanding the pathophysiology of gastrointestinal disorders, particularly IBD, including Crohn's disease and ulcerative colitis [126, 127]. Over time, the test gained popularity as a clinical tool for diagnosing and monitoring gastrointestinal disorders characterised by increased intestinal permeability, including malnutrition [125, 128]. During the test, individuals, with prior instruction of overnight fast, are given an oral solution containing both Lactulose and Mannitol. This solution typically contains a predetermined concentration of each sugar dissolved in water, which can vary depending on the study. Urine samples are then collected, usually over a period of 6 hours after consuming the solution. Finally, concentrations of Lactulose and Mannitol within the urine samples are measured, using mass spectrometry, to establish the ratio between both sugar molecules.

A high ratio of Lactulose to Mannitol indicates increased permeability of the gut epithelium, which may be indicative of conditions such as IBD, celiac disease or malnutrition [119, 125, 128, 129]. This is because lactulose, being the larger molecule, would normally have less passage through the intestinal barrier compared to mannitol. Lactulose and mannitol permeability test has also been used to assess gut permeability in young children with malnutrition and diarrhoea [125, 128, 130, 131], due to its non-invasive nature and simplicity of administration. However, Lactulose and Mannitol permeability test presents several limitations due to lack of standardization between studies and protocols [88]. Kosek Margaret et al. for example, showed that even in studies with uniform high-quality control strategies, such as the MAL-ED study, Lactulose:Mannitol results were not directly comparable [128]. Ordiz et. al. also questioned the reliability of the Lactulose:Mannitol ratio as a measurement of gut permeability and suggested the use of Lactulose solely as a marker of gut health [125]. In addition, recent studies have demonstrated that baseline mannitol levels can be affected by environmental contamination, due to mannitol being present in many foods, medications and skin products [88, 132].

2.2.2.2 Lactulose:Rhamnose permeability test

Rhamnose is an alternative to the use of mannitol, as it has similar molecular weight (164.15 Da), and is not commonly present in food, which minimizes the probabilities of contamination of the samples [132–134]. The Lactulose:Rhamnose (L:R) permeability test has also been used extensively in both clinical settings and research studies to assess gut permeability conditions. In clinical practice, L:R test has been used to diagnose conditions such as IBD and celiac disease and it has also been used to assess intestinal permeability in malnutrition studies in children [127, 132, 135–137].

Overall, dual sugar permeability tests, such as Lactulose:Mannitol or Rhamnose, have proved to be valuable tools for assessing gut permeability and identifying pathologies associated with increase intestinal permeability. However, despite their potential utility and the non-invasiveness of the approaches, it is essential to acknowledge the limitations of such tests. The use of L:M ratio (and

hence L:R ratio) was initially suggested to compensate for differences in the intestinal transit time and intestinal surface area, renal/hepatic function and total urinary excretion [125, 138, 139]. The ratio between these two molecules (Lactulose:Mannitol or Rhamnose) is then clinically used as a measure of gut permeability. However, the use of different sugar molecules and concentrations, and variations in the urine collection times or solution administration have considerably complicated the comparability of the results between studies, as demonstrated by Ref. [140–142].

Although, dual sugar permeability test, such as L:M and L:R are widely used in the clinic, the lack of standardisation precludes the meaningful comparison between pathologies and populations [88]. In addition, the clinical uptake of these tests is limited due to being challenging to perform in infants and in patients with reduced urinary output [143, 144]. In addition, the need for laboratory-based examination of the collected samples, often unavailable in low-resource settings, leads to higher costs and a delay between administering the tests and reporting results [88]. These disadvantages demonstrate the need for non-invasive, reliable and rapid diagnostic tools to provide improved assessment and monitoring of intestinal permeability (and other aspects of gut function) [36, 89].

2.3 The need for better assessment of gut barrier integrity

As we have explained previously, changes in intestinal function, particularly gut permeability, are known to be altered in wide-ranging gastrointestinal (GI) conditions including inflammatory bowel disease (IBD), coeliac disease and malnutrition, and also in conditions outside the GI tract such as schizophrenia, autism and Parkinson's disease [27–29, 143]. Endoscopic biopsies and histopathology, together with permeability tests such as Lactulose:Mannitol or Rhamnose assays and biomarkers, are the techniques currently used to assess permeability in the clinic [88]. However, our previous discussion has demonstrated that clinical use of tests such as endoscopic biopsies or dual sugar permeability tests have thus far been limited as they are either too cumbersome, too invasive, or due to a lack of standardisation in how the tests are performed. As a result, improved diagnostic tools that provide non-invasive, reliable and rapid measurements of intestinal permeability (and other aspects of gut function) have the potential to introduce considerable clinical benefits [36, 89]. This will be especially useful in low-income regions with limited resources and in children with additional associated risks.

Recent studies in both humans and animals have demonstrated the use of fluorescence spectroscopy – together with the use of fluorescence contrast agents – to non-invasively assess gut permeability and other physiological function in the clinic [90, 145–149]. To further understand the potential of non-invasive techniques for the assessment of gut permeability, the following chapter further explores the use of fluorescence spectroscopy, and more specifically transcutaneous fluo-

rescence spectroscopy, as a non-invasive, affordable approach for measuring gut permeability in patients with intestinal conditions, including malnutrition.

Chapter 3

Fluorescence Spectroscopy for the assessment of gut permeability

As discussed in Chapter 2, the gastrointestinal tract plays an important role in the wellbeing of the entire body, from nutrient absorption to hormone segregation and immune response. It is, therefore, involved in many health disorders such as coeliac disease, inflammatory bowel disease, and malnutrition [27, 29, 36, 143, 150]. Current techniques to assess and monitor gut function, particularly gut permeability, include endoscopic biopsies and permeability tests, such as Lactulose: Mannitol or Rhamnose [3, 15, 88]. However, these techniques are either highly invasive, unstandardised, do not allow a complete diagnosis and are difficult to perform in infants or patients with urinary problems [88, 143, 144]. Moreover, the mechanism and interactions behind the role of the GI tract in many of the above conditions and its possible repercussions are still not well understood.

The use of fluorescence spectroscopy, in conjunction with fluorescence contrast agents, has proved useful for the non-invasive assessment of gut permeability and other GI functions [90, 145, 148, 151]. In this chapter, we outline the basics of optical sensing, particularly fluorescence spectroscopy and the use of fluorescence contrast agents, together with the application of this technique for the assessment of gut permeability. Transcutaneous fluorescence spectroscopy is particularly relevant to this thesis as it is the optical technique implemented in the following chapters for the development of a sensor as a non-invasive, affordable approach for measuring gut permeability in patients with intestinal conditions, including malnutrition.

3.1 Fluorescence Spectroscopy

3.1.1 Photoluminescence

Optical sensors are based on the principle of converting light, or changes in light, into an electrical signal. In this thesis we focus on fluorescence spectroscopy. Fluorescence involves the emission of light, following a prior excitation and absorption process.

During the process of photoluminescence, which includes fluorescence and phosphorescence, a spontaneous emission of light is produced after the molecules are first excited by the absorption of light (Fig 3.1A) [152, 153]. When molecules are excited with a specific light wavelength, the molecules or atoms absorb photons, which cause the electrons to pass from their ground state to higher “excited” energy levels. Energy is then released sometimes in the form of light emission when the electrons return to their ground level. This emitted light can be differentiated into fluorescence or phosphorescence depending on the relaxation process that occurs when electrons return to their ground level [152, 153]. Fig 3.1A represents the process of phosphorescence and fluorescence along with other possible transitions in a typical fluorescent molecule.

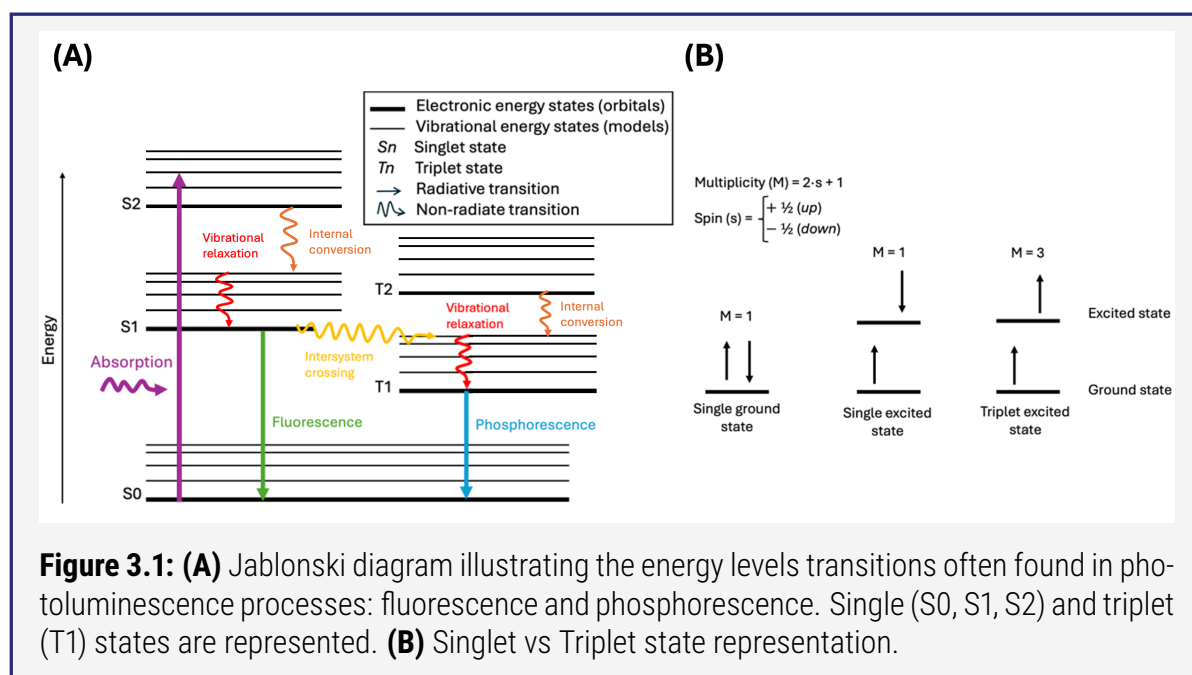


Figure 3.1: (A) Jablonski diagram illustrating the energy levels transitions often found in photoluminescence processes: fluorescence and phosphorescence. Single (S0, S1, S2) and triplet (T1) states are represented. (B) Singlet vs Triplet state representation.

Electrons present in the atoms have a specific spin associated with them (Fig 3.1B). Generally, these electrons paired themselves with electrons with opposite spins, resulting in a singlet state (S) with net spin equal to zero. However, in some cases, electrons with the same spin can be paired, resulting in a triplet state (T).

Fluorescence involves the transition between two electronic states of the same spin multiplicity (i.e., singlet state, S). When a molecule absorbs the external energy, one of the paired electrons transitions to higher electronic states, such as second or first electronic singlet state (S1 and S2,

respectively), while the other electron remains in the ground singlet state S_0 . This transition only occurs for fluorescent molecules. For these molecules, the excitation of the electrons is wavelength-specific (λ) and can be caused by the absorption of a single photon or by simultaneous absorption of several photons, known as multiphoton absorption. The probability of excited electrons returning from the singlet state to the ground state S_0 is high, resulting in short decay times of around 10^{-9} to 10^{-7} seconds [152–155]. Transitions from higher excited singlet states, such as S_2 are also possible and often include other non-radiative processes that will be explained later. The return of the excited electron to the singlet ground state produces a release of energy in the form of fluorescence light. Generally, this transition to the ground state involves less energy than the original absorption, resulting in longer wavelengths than the original excitation light ($\lambda_1 < \lambda_2$). In addition, the emission of fluorescence light can generally be prolonged as long as the excitation light continues.

In the case of *Phosphorescence*, electrons transit between states of different spin multiplicity, such as triplet-singlet states. This means that an excited electron, instead of transitioning from a singlet electronic state, such as S_1 , to the singlet ground state S_0 , it transits to a triplet state (T). In a triplet state, the excited electron is no longer paired with the electron in the singlet ground state, as they have now the same spin orientation (i.e., net spin is not zero). This triplet state is considerably more stable than the higher singlet states and prevents the rapid return of the excited electron to lower energy levels. The transition back to the singlet ground state occurs over a longer timescale, with decay times between microseconds and minutes [152–155] and requires a spin change of the excited electron.

As illustrated in Fig 3.1, other non-radiative processes often take place in addition to fluorescence and phosphorescence. The transition of an electron from the singlet ground state to higher energy states, such as S_1 and S_2 , occurs by absorption of radiation with specific wavelengths. Electrons that are in an excited state typically release energy non radiatively in the form of heat or transitioning down to the lowest vibrational energy level within that state, before returning to the ground state S_0 . This process, known as vibrational relaxation, involves the release of small amount of energy in a very short period of time. Electrons can also transit from higher electronic states to lower states without the emission of light. This phenomenon, known as internal conversion, can occur between singlet-to-singlet or triplet-to-triplet states. The efficiency of internal conversion increases as the distance between the energy levels decreases. Finally, intersystem crossing involves the transition of electrons between electronic states of different spin multiplicity, this means single-to-triplet state (S_1 to T_1 on Figure 3.1A). This transition involves the change of spin of the excited electron, resulting in excited and ground electrons with the same spin orientation (unpaired electrons). This can then lead to a phosphorescence emission of light when the excited electron returns to the ground state, as explained previously [152–155].

3.1.2 Properties of fluorescence molecules

In general, fluorescence molecules, or fluorophores, have specific properties associated to, for example, their molecular size, excitation and emission spectra, photostability and quantum yield [154, 155]. A narrow emission spectrum with well-defined peak wavelengths allows the emission of fluorescence light within a specific range of wavelengths with high precision, minimising spectral overlap with other fluorophores or background signals. In addition, the distance between the excitation and emission wavelengths, referred to as the Stokes shift, can also have implications on the detection of the fluorescence signals and can contribute indirectly to the self-quenching process. This phenomena, self-quenching, occurs when the fluorescence intensity emitted by the fluorophore is reduced due to interactions with other molecules of the same fluorophore type, which typically occurs at high concentrations [154, 155].

Another characteristic of fluorescent molecules is the quantum yield (ϕ). It represents the efficiency with which a molecule converts absorbed photons into emitted photons (fluorescence or phosphorescence). Consistent with this idea, quantum yield quantifies the probability that an electron in an excited state undergoes a radiative transition (fluorescence or phosphorescence) and emits a photon, relative to all possible transitions, including non-radiative processes. This measure will be important in the future chapters, specifically Chapter 7, when comparing between different fluorescence dyes. Mathematically, quantum yield is expressed as the ratio of the number of emitted photons to the number of absorbed photons and values range between 0 (no emission) to 1 (every absorbed photon results in an emitted photon) [153, 154]. The following equation describes this:

$$\phi = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}} \quad (3.1)$$

3.1.3 Endogenous and exogenous fluorophores

The properties presented previously have been described extensively over the past years for different fluorescence molecules. Some natural occurring molecules in the body have the capability of producing fluorescence signals when illuminated by a specific wavelength [153–155]. The fluorescence generated by these endogenous fluorophores is commonly referred to as autofluorescence and some examples include Nicotinamide Adenine Dinucleotide (NADH), Flavine Adenine Dinucleotide (FAD), elastin and collagen, which are significantly present in many connective tissues [153, 156, 157]. The fluorescence of some of these molecules, and its intensity, is often dependant on their metabolic state or the interactions with other molecules. Because of this, the autofluorescence of these molecules have been used in some cases to study differences between normal and diseased tissues [153, 157, 158]. Riboflavin, or vitamin B2, for example, is a micronutrient with intrinsic fluorescence that plays an important role in a wide range of cellular processes [159, 160]. Although vitamin B2 is not synthesised in the body, it is naturally present in our system through

intestinal absorption (diet) and it has been used to study intestinal permeability [161, 162].

However, the detection of the fluorescent signal from autofluorescence molecules is challenging because within the body, and even on a cellular level, there are multiple endogenous fluorophores that could emit light simultaneously when excited by specific wavelengths. This induces a non-specific background signal that can potentially impact the specificity and accuracy of the measurements [163, 164]. In addition, because some endogenous fluorophores are dependent on the diet or metabolic state, autofluorescence signals might change over time and between individuals making them unreliable and difficult to compare between populations.

Exogenous fluorophores, on the contrary, are molecules not naturally present in biological tissues [154, 155]. They are typically synthesised or administered for specific imaging purposes. In this section, we will focus on three widely used fluorescence contrast agents: FITC-dextran, fluorescein and Methylene blue.

Fluorescein isothiocyanate (FITC) dextran is a fluorescence contrast agent commonly used in research for the assessment of vascular permeability, cell permeability and tissue barrier function [165–169]. Its structure consists of a dextran polymer chain conjugated with fluorescein isothiocyanate (FITC) molecules.

FITC, a synthetic derivative of fluorescein, is a fluorescent label widely used due to its high fluorescence quantum yield (0.91) and stability under various experimental conditions [170]. Since the early 1960s, researchers began conjugating FITC to various molecules, including antibodies, proteins and carbohydrates, to create fluorescent probes for the study of in vitro and in vivo processes, such as vascular circulation [171, 172].

Dextran, a complex polysaccharide derived from microbial fermentation of sucrose, was identified as a suitable carrier molecule for FITC due to its compatibility, water solubility and ability to form stable conjugates [173]. In the 1960s and 1970s, scientists began using FITC dextran conjugates of various molecular weights, which can go from 4 kDa to 2000 kDa, in studies of vascular physiology and tissue permeability [165, 168, 169]. The excitation and emission wavelengths of FITC dextran are approximately 490 and 520 nm, respectively. Its wide versatility allowed FITC dextran to become a valuable fluorescence contrast agent for the study of physiological and pathological processes, such as inflammation, angiogenesis, gut barrier function and drug delivery [34, 166, 169, 174, 175]. Due to the large size of the conjugated dextran, FITC dextran is unable to pass through the mucosal intestinal barrier in normal physiological conditions. This allowed researchers to use FITC-dextran as a marker of gut barrier dysfunction in vitro and in vivo studies. In addition, both, plasma and urinary recovery of FITC-dextran have demonstrated to be a valuable approach to study the function of the gut permeability in pathological and physiological conditions [90, 166, 176, 177].

Another fluorescence contrast agent widely used in the clinic is fluorescein sodium. Fluorescein is a

synthetic organic compound that is known for its strong orange-red colour in its powder form with a high yellow/green fluorescence intensity emitted at around 520 nm when excited by a light with a wavelength of 488 nm [178]. Fluorescein sodium was first synthesised by Adolf von Baeyer in 1871 and its fluorescence properties led the use of fluorescein into medical diagnostics and research, as well as in industry [179–181]. It has a molecular weight of 332.20 Da and a quantum yield similar to FITC dextran of 0.92. Contrary to FITC dextran, fluorescein is approved for medical use. In the clinic, fluorescein sodium is extensively employed as a fluorescent marker for diagnostic purposes, particularly as an intravenous or oral administration for ophthalmic angiography for assessing retinal blood flow, identifying retinal vascular disorders and detecting ocular abnormalities such as corneal lesion [182–188]. Due to the widely use of fluorescein sodium in the clinic, adverse events associated with fluorescein has been studied and reported [182]. The most common adverse event is nausea and is associated with approximately 1 out of 10 patients. Other more severe adverse events, such as anaphylaxis or fatal allergic reactions have much lower occurrence, with 1 out of 2000 or 1 out of 2000,000 patients. In addition, fluorescein's ability to highlight blood vessels and other structures has also extended its use to various surgical procedures and endoscopic imaging applications [189, 190]. The relative smaller molecular size of fluorescein, in comparison with FITC dextran, allows fluorescein to permeate cell membranes and the epithelial barrier, leading to both *in vitro* and *in vivo* studies of the assessment of barrier function.

In research, another well known fluorescence contrast agent is Methylene Blue. It is a synthetic compound with a distinctive blue colour that has extensively been used in research and in the clinic due to its various properties. Methylene Blue, with a molecular weight of 319.85 Da, emits fluorescence light at 690 nm when excited by a wavelength of 665-670 nm. Since the late 1870s, when it was first synthesised by Heinrich Caro, the use of Methylene Blue expanded into medicine, particularly in the treatment of malaria, but also as a diagnostic tool for sentinel lymph and parathyroid mapping [180, 191–195]. The ability to penetrate membranes allows its use in medical imaging for cancer removal, and it has also been used in animal models for *in vitro* evaluation of epithelial permeation [196]. Similar to fluorescein, Methylene Blue is clinically approved and therefore its adverse reactions have been investigated. This includes, dizziness, headaches or respiratory and vascular problems, particularly important for children. [194, 197, 198]. Despite its adverse events, Methylene Blue is still in use in the clinic and research as a fluorescence contrast agent [199–201].

3.1.4 Transcutaneous fluorescence spectroscopy

As we have discussed in the previous section, the study of the optical properties of endogenous and exogenous molecules have been used to investigate different physiological and pathological conditions. Spectroscopy, and particularly fluorescence spectroscopy, is the technique commonly used to analyse the fluorescence light emitted by these molecules both *in vitro* and *in vivo* [153–155].

Spectroscopy is a technique to study the interaction between matter and electromagnetic radiation, such as light, as a function of wavelength, providing valuable information about the composition, structure and properties of materials. When light interacts with a tissue, it can undergo different optical processes, such as absorption, scattering and/or reflection. The absorption of light can also induce the emission of light, such as fluorescence or phosphorescence. The resulting spectrum from each of the optical processes can reveal important characteristics of the sample and so different spectroscopic techniques have implemented them to investigate specific physiological and pathological processes.

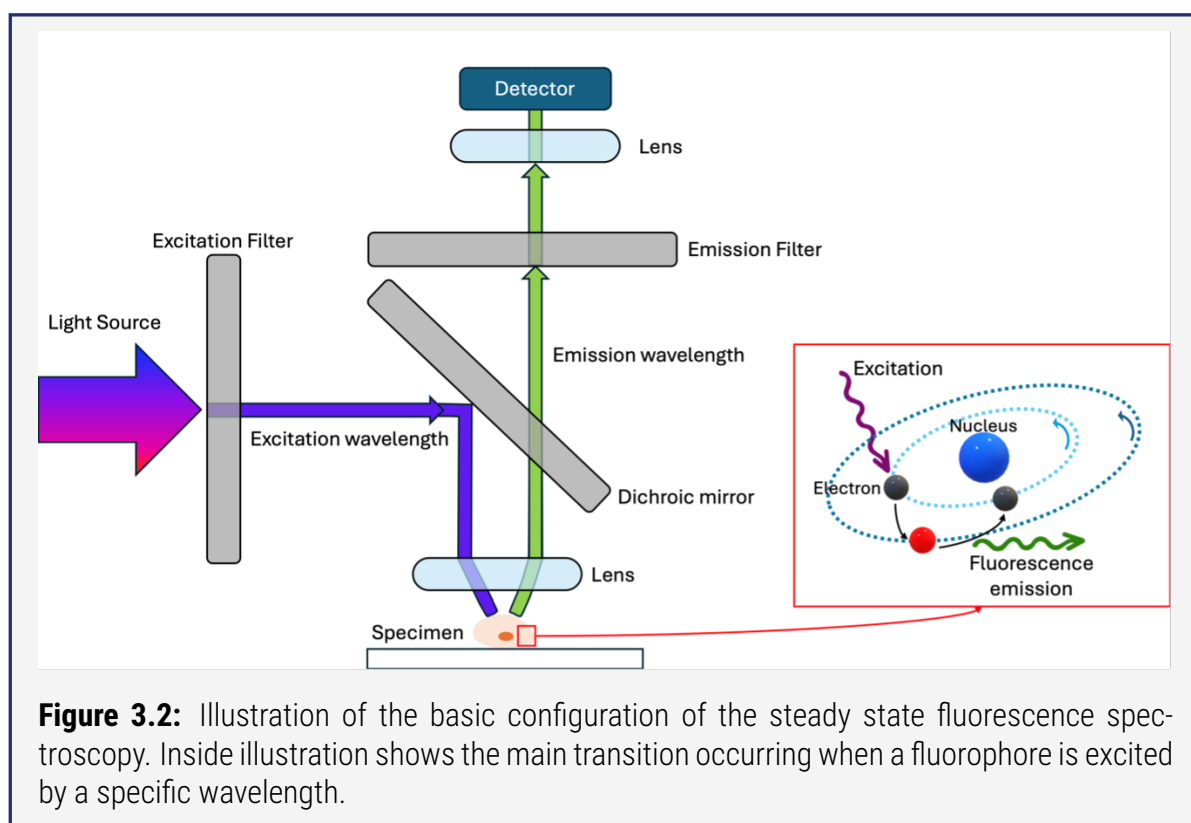
Reflectance occurs when light is reflected directly back from the surface of the sample and is typically used in reflectance spectroscopy to study the surface properties of materials. With biological samples, light can also undergo multiple optical processes at the same time, including both scattering and absorption, before being reflected back to the surface [153, 154]. In these cases, a portion of the light is directly reflected from the surface (specular reflection), whereas the rest of the remaining light scatters into the tissue and undergoes numerous internal absorption, reflection and scattering events (diffuse reflection or elastic scattering) before returning back to the tissue surface. The light reflected back from the deep sections of the tissue, measured by diffuse reflectance spectroscopy (DRS), provides information about the absorption and scattering properties of the tissue sample.

Scattering and absorption are an integral part of fluorescence spectrometry, as how the light interacts with the sample influences the excitation and emission of fluorescence from the specimen. As we have discussed previously, the absorption of light is essential for initiating the fluorescence process to promote excited electrons to higher energy levels. Fluorescence spectroscopy is a technique that measures the fluorescence light emitted by fluorophores previously excited by a light with a specific wavelength. Because the excitation and emission light beam normally travel through the tissue sample, they undergo elastic scattering events within the specimen that can have a large influence in the resulting fluorescence signal intensity. This is especially important when investigating molecules deeper in the tissue. Fluorescence spectroscopy systems are built to reduce these background signals as much as possible or any other artifacts and to maximise the fluorescence signal emitted by the fluorophores. Steady state fluorescence spectroscopy and time-resolved fluorescence spectroscopy are two different techniques used to study the different characteristics of fluorophores. Time-resolved fluorescence spectroscopy focuses on measuring the decay rate of fluorescence intensity after excitation of the fluorophore, producing complex three-dimensional (3D) data, including time and spectral information. For this thesis, steady-state fluorescence spectroscopy is particularly interesting as it provides real-time information about the fluorescence intensity, which can directly correlate with fluorophore concentration at the designated measurement site.

In steady state fluorescence spectroscopy, the sample is continuously exposed to a steady exci-

tation light source, usually using a narrowband source such as a laser or a lamp. The excitation light excites the molecules within the sample to higher energy levels, leading to the emission of fluorescence light. This fluorescence light is then measured using a spectrometer, which separates the emitted fluorescence light into its component wavelengths, or a photodetector, which measures the total fluorescence intensity. Fig. 3.2 shows a schematic representation of a typical configuration used in fluorescence spectroscopy.

As shown in the image 3.2, the sample is excited with a continuous and stable light source that emits a specific wavelength and causes the fluorophores within the sample to emit fluorescence light. Optical filters are normally used to selectively transmit certain wavelengths, while blocking others, to match the absorption spectrum of the sample. Lenses or mirrors are also used to focus and guide the excitation light directly onto the sample. After excitation, the sample emits fluorescence light in all directions. Lenses and mirrors, as well as optical filters, are again used to focus the emitted fluorescence light onto the detector and to isolate specific fluorescence emitted wavelengths for the analysis.



The fluorescence signals generated by the fluorophore can be analysed either by wavelength or emission spectrum, representing the distribution of fluorescence intensity as a function of wavelength using a spectrometer, or by intensity. This last case is of high interest for the assessment of changes in gut permeability as the fluorescence intensity is linearly correlated to the concentration of the fluorophore (within concentration ranges in which self-quenching is negligible). In addition, due to the configuration and the characteristics of the technique, steady state fluorescence spec-

troscopy can also be used to non-invasively measure fluorescence signals through the skin. This is known as **transcutaneous fluorescence spectroscopy** and is the technique further investigated in the following chapters.

3.2 Applications of fluorescence spectroscopy for the assessment of the gut barrier

Fluorescence contrast agents, together with fluorescence spectroscopy systems, have been used in many studies for the assessment of gut barrier function. The approach entails the oral or intravenous administrations of the contrast agent and the following measurement of the permeation of this fluorescence molecule through the gastrointestinal epithelium as an assessment of the gut barrier function. Most common fluorescence contrast agents used include fluorescein and fluorescein-based probes such as FITC dextran. In the case of FITC dextran, multiple studies, particularly in animal models, have shown the potential of this fluorescence compound as a marker in plasma for gut barrier function [166, 176, 177, 202–205]. Boonhai et al and Purandare et al, for example, measured the concentration of 4 kDa FITC-dextran in plasma from mice with and without gut barrier dysfunction and demonstrated an increase in the permeation of FITC-dextran on mice with leaky guts [202]. These studies are based on the fact that FITC-dextran, due to its relatively large molecular size (i.e., 4 kDa to 70 kDa commonly used), permeates more easily in conditions where the tight junctions are compromised. Although, multiple studies have proved FITC-dextran to be a valuable plasma marker of gut permeability, high doses of FITC-dextran are needed to achieve a good signal level - as the number of FITC molecules per dextran chain is typically low (<1:1) to avoid self-quenching. This shows that the use of FITC-dextran can be relatively costly, which can limit its clinical application in large populations and low-income settings [205].

Fluorescein is a more affordable fluorescent contrast agent that has also been widely used for the assessment of gut barrier function [90, 188, 190, 206]. As discussed previously, fluorescein was initially approved as a contrast agent used in angiography [185–188]. However, it was quickly integrated in multiple approaches due to its safety and molecular properties. In confocal laser endomicroscopy, for example, fluorescein is the preferred contrast agent to use [190, 207]. In this case, fluorescein is intravenously injected, and the gut permeability is assessed by measuring the permeation of the fluorophore from the plasma into the lumen. Consistent with this idea, Dorshow et al. and Debreczeny et al. demonstrated that gut permeability can also be evaluated by measuring the concentration of fluorescein in plasma and urine after oral administration [148, 151]. More interestingly, recent studies in both humans and animals have demonstrated that transcutaneous fluorescence spectroscopy of orally ingested fluorescein has the potential for non-invasive monitoring of gut permeability and other gastrointestinal functions in the clinic [90, 145, 148]. This technology has the potential to produce clinical results within hours rather than days (as data can

be analysed immediately without requiring laboratory analysis) and does not require collection of urine, blood or stool samples (unlike other technologies such as L:M or L:R tests). This approach is covered in more detail in future chapters.

Transcutaneous fluorescence spectroscopy, together with the use of other fluorescence dyes, has also been integrated into compact and wearable sensors, such as TGFR and Preclinical MX developed by MediBeacon, for the measurement of kidney function (i.e., glomerular filtration rate) [149, 208]. However, to date, transcutaneous fluorescence spectroscopy to measure gut permeability has only been deployed clinically using laser-based spectrometers and microscopes (as the one developed by Dr Thompson and hereafter referred to as benchtop system, Fig. 4.1), which are both large and expensive [89, 90, 146, 209]. The use of these benchtop systems can therefore introduce additional challenges in certain patient groups (e.g. children/infants) and in certain environments (such as low-income countries), thereby precluding widespread deployment.

To address the above limitations, this thesis focuses on developing a compact fluorescence spectroscopy sensor for monitoring gut permeability using transcutaneous spectroscopy. The primary function of the device is to non-invasively detect fluorescence signals at the tip of the finger as an orally ingested contrast agent (fluorescein) permeates through the gut barrier into the blood stream. The aim of the project is to further develop the technique towards clinical use for the validation of the technology in different gut permeability conditions, including undernutrition. Chapter 4 presents the development of an algorithm for the processing and analysis of the fluorescence intensity signals collected from the transcutaneous fluorescence system (previously presented as benchtop system). The findings from this chapter provide information about the features relevant to characterize the fluorescence signals that can be used to guide the development of improved systems.

Chapter 5 of this thesis is then centered on the development of a more compact and affordable optical system suitable for deployment in clinical settings globally, including low-income regions. This new system is aimed to improve some of the limitations that are present on current techniques such as dual sugar permeability tests or, the previously mentioned, benchtop system. In Chapter 6, this device is then implemented in an in vivo study to further investigate the changes in gut permeability characteristics in an undernourished population. Finally, based on the results obtained from chapter 5 and 6, Chapter 7 focuses on investigating in cellular models the use of other fluorescent dyes for the assessment of the gut barrier. Altogether, the work presented in this thesis aims to further develop a technique that could help to overcome some of the limitations that current clinical applications have for the assessment of gut barrier function with the ultimate goal to further understand the mechanism behind the changes in gut permeability associated with some pathological conditions, including undernutrition.

Chapter 4

Automatic processing and analysis of fluorescence spectroscopy data - Proof-of-concept

As discussed in Chapter 2, current techniques for monitoring and diagnosing abnormal changes in gut permeability rely on the use of techniques, such as endoscopic biopsies, sugar permeability test and haematological markers, that are often limited due to their invasiveness, lack of standardisation and complexity [90, 143, 144]. To address these challenges, a novel technique using transcutaneous fluorescence spectroscopy for non-invasive assessment of gut function was developed by Dr Alex Thompson and colleagues [90, 145]. Previous studies showed promising results using this approach to measure gut permeability and gastric emptying rate [90, 145]. However, gut permeability measurements were made in a very limited cohort of participants [145]. This chapter aims to further demonstrate the potential of this technique by reporting the results of a proof-of-concept algorithm to automatically process and analyse fluorescence spectroscopy data collected from an *in vivo* study. This study involved the use of the optical fibre spectrometer system, developed by Dr. Alex Thompson and colleagues [90, 145], and was carried out in collaboration with Dr. Jonathan Gan, who performed the *in vivo* fluorescence data collection at St Mary's Hospital in London. The findings on this chapter also served to illustrate the potential of the data collected with the fluorescence system for monitoring and diagnostic changes in gut permeability. The work presented in this chapter has been published on Methods and Applications in Fluorescence, [209] and is used for the data analysis in subsequent chapters.

4.1 Introduction

Intestinal permeability is a functional feature of the intestinal wall that defines the degree to which molecules can pass from the intestine into the blood stream at given sites [210]. Two aspects of gut permeability that can be measured are transcellular permeability (representing passage through the water pores of cell membranes) and paracellular permeability (passage through the tight junctions between cells in the intestinal wall) [211, 212]. Paracellular permeability is known to be altered in wide-ranging gastrointestinal (GI) conditions such as coeliac disease, inflammatory bowel disease (IBD) and environmental enteric dysfunction, and in conditions outside the GI tract such as schizophrenia, autism and Parkinson's Disease [143]. Thus, for the practising clinician, a reliable tool to measure gut permeability could potentially provide a new way to diagnose these conditions and monitor the effectiveness of treatments. Specific to the GI area, such a technique could provide improved monitoring of treatment responses and could also help in developing a better understanding of functional gut disorders (a 'catch all' term for poorly understood gastrointestinal conditions that are often treated as a diagnosis of exclusion).

As explained in Chapter 2, at present, there are multiple options available to measure gut permeability, including dual sugar permeability tests (L:M and L:R), haematological and bacterial markers and endoscopic biopsies [142]. However, clinical use of these tests has thus far been limited [36, 142], exacerbating the need for improved technologies to assess gut permeability [36, 89, 90, 145, 146].

To address this challenge, we have developed a novel technique – transcutaneous fluorescence spectroscopy – for non-invasive assessment of gut function using orally ingested fluorescein [89, 90, 145, 146]. Fluorescein is a fluorescent contrast agent that is inert, safe and has been used in many clinical settings such as routine ophthalmology procedures (e.g. fluorescence angiography) [185] and confocal laser endomicroscopy during colonoscopy [190]. Furthermore, the potential of fluorescence spectroscopy in gut permeability assessment (using fluorescein or other contrast agents) has been demonstrated both in cells and in animal models [148, 206, 213–215].

Using transcutaneous fluorescence spectroscopy, gut function can be assessed non-invasively through the following process: patients consume an oral dose of fluorescein (or another fluorescent contrast agent), and a wearable probe/sensor is used to detect the fluorescence signal through the skin (*transcutaneously*) as the contrast agent permeates from the gut into the blood stream. As such, it is possible to measure fluorescein uptake from the gut into the blood stream in real time. Appropriate analysis of the resulting fluorescence vs. time curve then allows quantification of multiple elements of GI function including gastric emptying rate and gut permeability.

We have recently demonstrated the potential of this technique to monitor gut function and to measure gastric emptying rate in studies in healthy volunteers. Lett et al. demonstrated the use of transcutaneous spectroscopy to measure gastric emptying rate as a feasible alternative to the

conventional paracetamol absorption test [145]. Maurice et al. showed promising preliminary results using the method to measure gut permeability (albeit in a very limited cohort of participants [90]). Importantly, both studies showed that the test is well tolerated and has the potential to produce clinical results within hours rather than days (as data can be analysed immediately rather than requiring analysis of samples in a laboratory). Furthermore, the approach is non-invasive and does not require collection of urine, blood or stool samples (i.e. unlike competitor technologies such as L:M or L:R tests and endoscopic biopsies).

4.1.1 Aims

To further demonstrate the clinical potential of transcutaneous fluorescence spectroscopy for non-invasive assessment of gut permeability, fluorescence data was collected from a clinical experiment in 11 healthy volunteers. All participants took part in two repeat experiments in which they ingested fluorescein dissolved in water (day 1) and fluorescein dissolved in a concentrated aqueous sugar solution (day 2). Ingestion of concentrated sugar solutions is known to drive temporary increases in gut permeability [216, 217]. Previous studies have showcased the potential of transcutaneous fluorescence signals to provide information about gut permeability [90, 146]. However, limited analysis of these signals has been investigated. This chapter presents the fluorescence signals collected for each participant and experimental condition and focuses on developing and analysing different data processing approaches to investigate the potential of the technology - transcutaneous fluorescence spectroscopy - to non-invasively discriminate between two differing states of intestinal permeability. The analytical approaches investigated in this chapter centre on the extraction of relevant parameters from the fluorescence signal to accurately discriminate between different gut permeability stages.

4.2 Methods

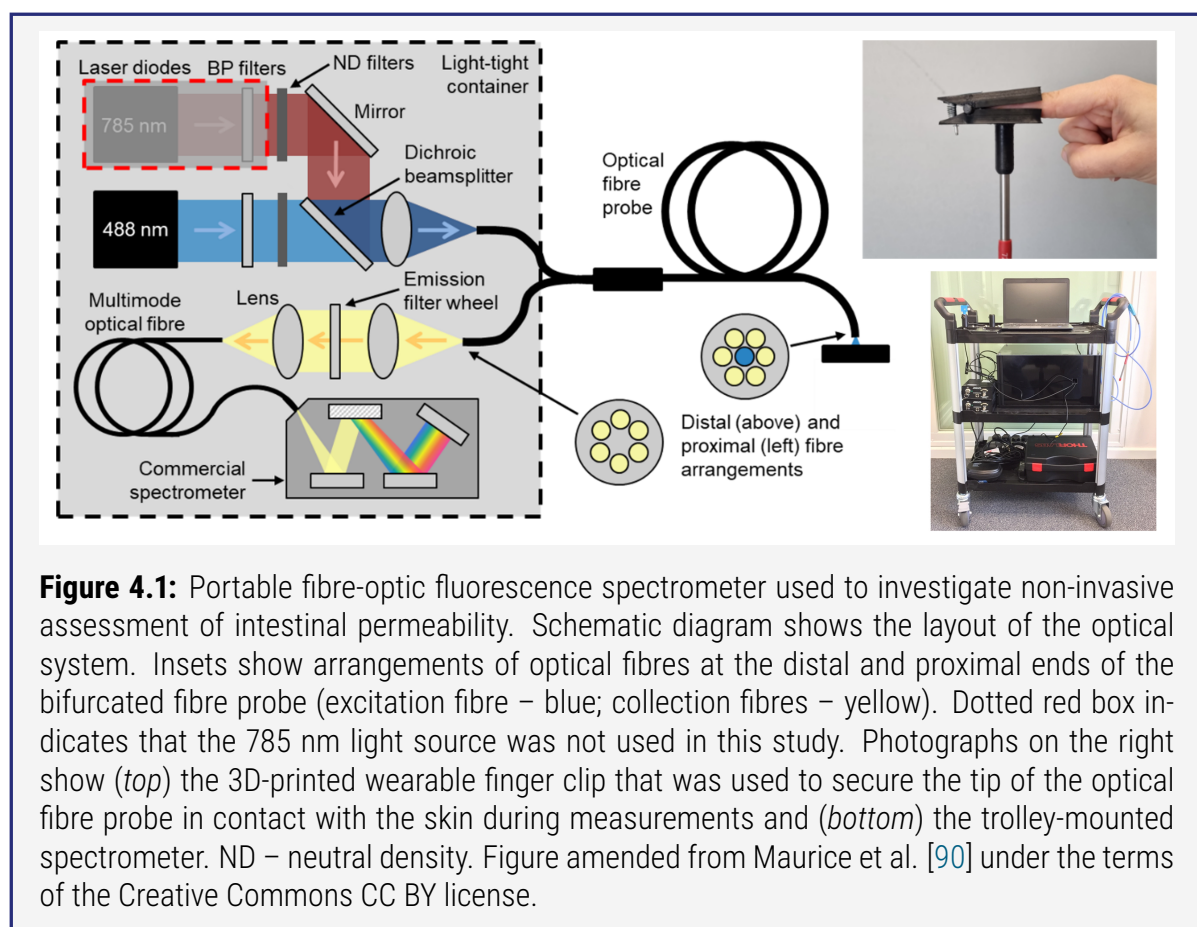
4.2.1 Fibre-optic spectrometer

Following oral ingestion of fluorescein, a fibre-optic fluorescence spectrometer was used to detect fluorescence signals at the fingertip. This system, developed by Dr Alex Thompson, has been described in detail in previous studies [90, 145]. Briefly, it consists of two laser sources (at 488 nm and 785 nm) that are coupled into a bifurcated fibre-optic probe. The fibre-optic probe delivers excitation light to the measurement site (the fingertip, where it is secured using a 3D-printed clip) and also collects the resulting fluorescence signal and delivers it to a spectrometer for detection. In this study, only the 488 nm laser was used, as this provided efficient excitation of fluorescein. The laser power (at 488 nm) at the tip of the fibre-optic probe was limited to 63 μ W using a combination of variable neutral density (ND) filters and the laser control software. This ensured that the laser exposure was below the maximum permissible exposure for the skin in all cases [150, 218, 219],

allowing safe use in the clinic. The optical system and 3D-printed finger clip are shown in Fig. 4.1. The entire system is controlled using a laptop computer running custom-written LabVIEW software and is housed within a wheeled trolley to facilitate use within clinical environments.

The spectrometer records both the fluorescence signal and the directly scattered laser signal (by switching between two filter ports in a motorised filter wheel). The backscattered laser signal is used to normalise the fluorescence data to correct for any variations in laser power or probe position between measurements. This normalisation procedure – which is described in detail in previous studies³ [90, 145] – then allows for calculation of a fluorescence vs. time curve (e.g. see Figure 4.2), which can be analysed to extract information regarding gut permeability and other elements of GI function.

For the clinical experiments reported here, the spectrometer was programmed to record the fluorescence and laser signals once every minute for a total of 3 hours. This allowed for calculation of fluorescence vs. time curves with sufficient temporal resolution to observe the dynamics of the fluorescein signal (e.g. the permeation from the gut into the blood stream and the elimination of the dye from the blood). In turn, this permitted calculation of a set of parameters designed to provide readouts of gut permeability (see more details in Data Analysis section below).



4.2.2 Clinical trial measurements

Clinical trial measurements were performed in healthy participants at St. Mary's Hospital (London, UK) between February 2021 and October 2021. Participants were recruited and consented in accordance with the clinical study protocol [220], which received ethical approval from the UK Health and Research Authority (HRA) and a local Research Ethics Committee (REC) (IRAS Project ID – 242462, REC reference – 18/LO/0714/AM04). Recruitment of participants and clinical measurements were performed by Dr. Jonathan Gan. This study was conducted in line with local Good Clinical Practice (GCP) guidelines and in accordance with the World Medical Association's Declaration of Helsinki. All participants gave informed consent prior to the study and all were screened before participation to confirm that they did not have a diagnosis of any known conditions that altered gut permeability. All participants were aged 18 and above and had no previous reactions to fluorescein. Participants who were pregnant, were breast feeding, or had taken antibiotics for the last 4 weeks were excluded.

After 6 hours of fasting, all participants ingested 500 mg fluorescein sodium (supplied as a 5 ml vial of 100 mg/ml fluorescein; Fluorescite Antera 10%) dissolved in 100 ml water. A dose of 500 mg was chosen as this was expected to provide strong signal levels in all participants (previous work demonstrated a limit of detection for orally ingested fluorescein of less than 25 mg [145]) and because it represented the clinically approved dose of fluorescein for other medical procedures. Fluorescence measurements were started immediately before ingestion of the fluorescein to allow collection of a baseline measurement. Measurements were taken using the fibre-optic spectrometer discussed above, with the tip of the probe secured in contact with the skin at the fingertip using a 3D-printed mount (see photograph in Figure 4.1). Spectra were then recorded at one-minute intervals for a total of three hours to allow investigation of the kinetics of the recorded fluorescence signals.

After a minimum of 7 days, participants were invited back to St. Mary's hospital for a repeat study, this time ingesting 500 mg fluorescein sodium with 60 g sugar in 100 ml water. Fluorescence data was collected in an identical manner to that described above. As discussed briefly above, the concentrated sugar solution is expected to have a hyperosmolar effect that drives a transient increase in gut permeability that is broadly comparable to the changes observed in coeliac disease [216, 217]. Preliminary results reported by Maurice et al. supported this [145]. Thus, we hypothesised that if we could differentiate between the data collected with and without sugar, this would indicate potential for non-invasive assessment of intestinal permeability. Data recorded using the fibre-optic spectrometer were therefore compared across the two study days.

A statistical power calculation was used to estimate that a minimum of 10 participants would be required in this study to obtain statistical power of over 80%. Thus, in total, 13 participants were recruited and included in the study to account for the possibility of participant dropout/withdrawal. Data from two participants was removed from the analysis as one participant was later found to

Table 4.1: Demographics of participants included in the study. BMI – body mass index (kg/m^2); M – male; F – female.

ID	Age	Sex	Ethnicity	BMI
P1	32	M	Caucasian	28
P2	50	M	Caucasian	31
P3	28	M	Caucasian	25
P4	31	M	Asian	30
P5	24	F	Caucasian	23
P6	28	M	Asian	25
P7	29	F	Asian	29
P8	33	M	Asian	26
P9	27	F	Asian	23
P10	28	M	Asian	28
P11	30	F	Asian	25

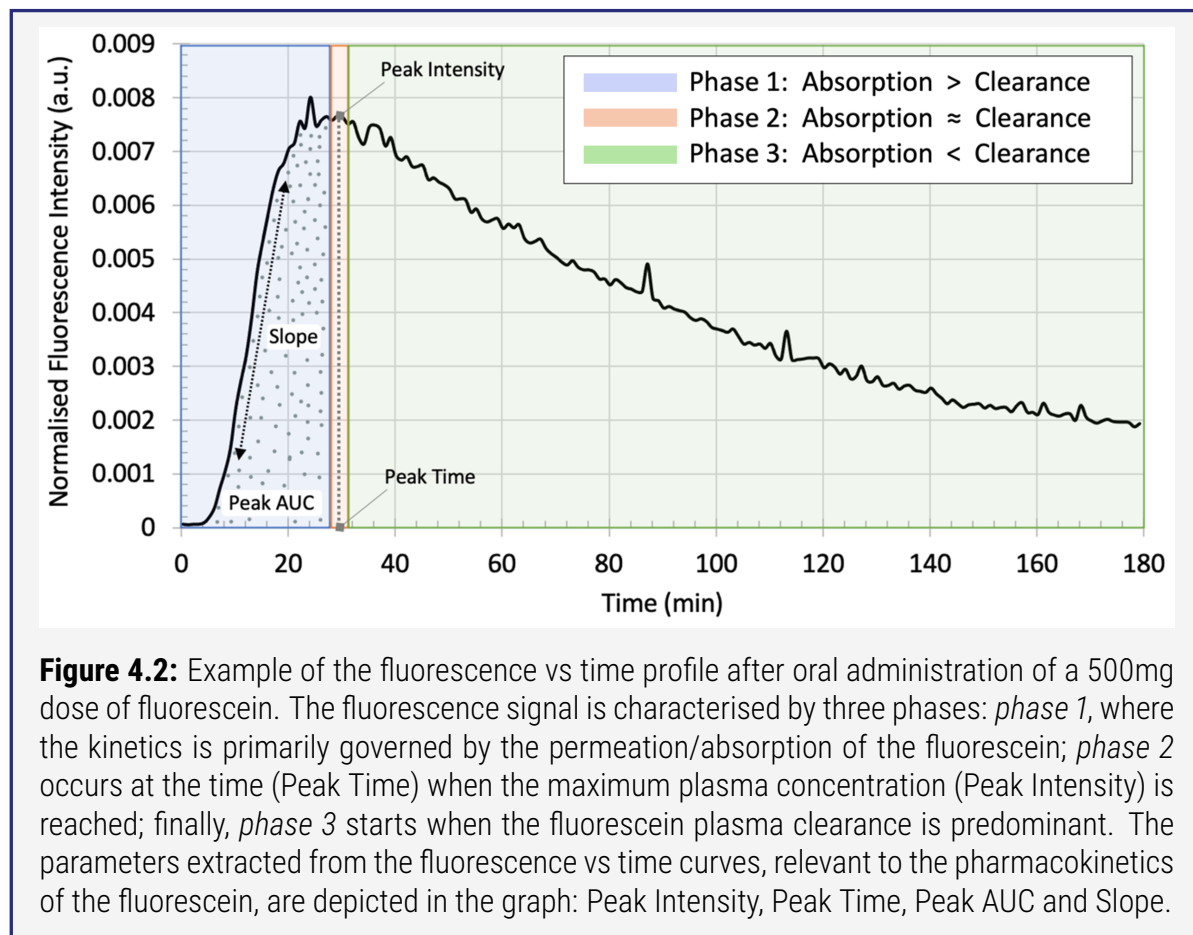
have a health condition that was expected to impact gut permeability and the second participant did not complete the second study day (meaning that their dataset was incomplete). Of the remaining 11 participants, 4 were female and 7 were male, and the median age (of all 11 participants) was 29 years (range: 24-50 years). All 11 participants included in the analysis had no previous conditions that would affect gut permeability and did not have any bowel symptoms. The participant demographics – age, sex, ethnicity and body mass index (BMI) – are tabulated in Table 4.1.

4.2.3 Investigation of different computational approaches for data analysis

To assess whether the transcutaneous fluorescence data could be used to differentiate between two differing states of intestinal permeability (i.e. between the fluorescein only data and the fluorescein + sugar data), different methods of data analysis were investigated. Briefly, the approaches focus on the extraction of parameters from the fluorescence signals in order to obtain features from the signals that will help to discriminate between the datasets.

As observed in previous studies, the pharmacokinetics of the fluorescein intensity-time profile is

generally characterised by three distinct phases, each influenced by the interplay of permeation/absorption and clearance processes of the fluorescein, as depicted in Figure 4.2.



In the initial phase, fluorescein is primarily concentrated in the gut, leading to greater rate of absorption from the gut to the bloodstream. During this phase, the elimination of the fluorescein from the bloodstream is predominantly lower than the absorption, resulting in a positive increase in the fluorescence intensity detected. As we approach the second phase, the permeation of fluorescein is gradually balanced with its clearance, culminating in a saturation point denoted as Peak Intensity. The subsequent third phase is characterised by a decrease in the fluorescence intensity signals driven by the prevailing elimination of fluorescein from the bloodstreams.

In addition, some physiological factors such as gastric-emptying rate and motility of the intestine may cause delay in the oral absorption of fluorescein. In such case, the delay translocation of fluorescein from the stomach to the gut can lead to slower permeation/absorption rates [221], therefore, affecting the time at which the saturation point is reached (denoted as Peak Time), as well as the average gradient within the first permeation phase (denoted as Slope). Following these considerations, the methods investigated in the following sections focused on the processing of the fluorescence signals for the extraction of different parameters that will help to discriminate between datasets.

The first analytical approach focuses on the extraction of parameters, such as Peak Intensity, Peak Time and Slope. These parameters could potentially serve as indicators of the rate of uptake of fluorescein and potentially be used to correct for changes in gastric emptying or other factors influencing fluorescein permeation to ultimately differentiate between gut permeability stages. In addition to that, subsequent sections will delve into more advance techniques, such as exponential decay fitting and phasor analysis, for the extraction of potential useful features from the fluorescence signals. Altogether, the approaches investigated in this chapter aim to provide a characterization of the kinematics of the fluorescein permeation, allowing for an effective assessment and comparison of different stages of gut permeability.

4.2.3.1 Automatic peak selection

The approaches investigated in this chapter analyse the fluorescence signals to extract and calculate robust features/parameters that will improve the discrimination of the two datasets. In addition to the parameters mentioned previously – Peak Intensity, Peak Time and Slope, the total fluorescein permeated from the gut to the bloodstream can also be characterised by the intensity detected up until the Peak Time. Therefore, the area under the curve up until the first saturation point (Peak AUC) could also be potentially useful for the analysis.

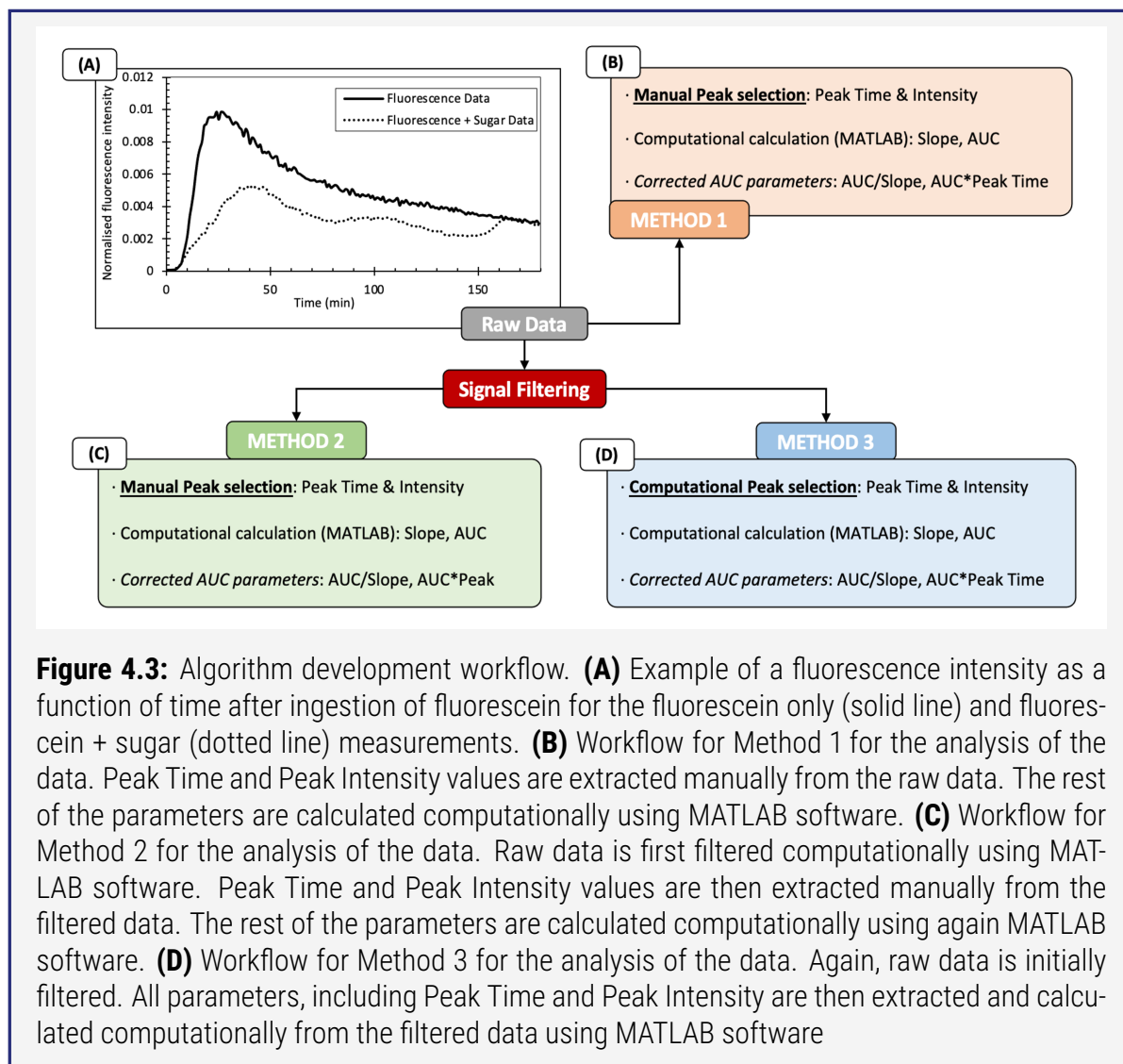
In this section, we extract different parameters from the fluorescence curves and most of the work focuses on optimising the accuracy of the identification of the parameter Peak Time, as the rest of the parameters (Peak Intensity, Slope and Peak AUC) are highly dependent on it. To do so, different approaches, classified from simple to complex, are investigated to integrate different levels of automation for the peak time selection. Figure 4.3 shows a representation of the workflow followed to extract the parameters in the three investigated approaches: *Method 1*, *Method 2* and *Method 3*.

The first method (Method 1) is the simplest and involves minimal automation. Manual inspection of the raw data identified the Peak time and the Peak Intensity values. The rest of the parameters, Peak AUC, Total AUC and Slope, were calculated computationally in MATLAB.

The second method (Method 2) implemented a median filtering step to reduce noise and provide more accurate identification of the Peak Time values. Peak times were once again determined manually, but this time using the filtered data. As Method 1, computational methods were used to extract the Slope and Peak AUC values using MATLAB. However, for the Peak AUC values, the manually identified Peak Times values (this time, based on the filtered data) were used in this calculation.

Finally, the third method (Method 3) involved automatic identification of the Peak Time values using the filtered data. In order to detect the Peak Time values, this 3rd method integrates a computational function to detect local maxima. Slope and Peak AUC values were again computationally calculated using the filtered signals, but this time, Peak AUC also used the Peak Time values ex-

tracted computationally from the filter signals. This approach served to reduce the risk of manual/observer bias in the identification of peak times, as no manual input was used, and provided fully automated analysis of the collected data.



4.2.3.2 Exponential decay fitting

As mentioned in Section 4.2.3, variations in the gastric emptying rates can impact the permeation of the fluorescein from the gut to the bloodstream. In addition to this, another factor, such as the clearance rate, can also influence the total fluorescence detected during the data collection. The elimination of the fluorescein from the bloodstream starts as soon as the molecule permeates into the vascular system. This means that the plasma concentration of fluorescein (after permeation from the gut) decreases with a rate known as the fluorescein renal and hepatic clearances, of ≈ 1.75 and $1.50 \frac{\text{ml}}{\text{min*kg}}$ [222]. Because of this, the fluorescein intensity measured at Peak Time does not represent the true total concentration of fluorescein permeated from the gut to the bloodstream, as part of it has already been eliminated. To accurately describe and correct for the elimination

of the fluorescein from the bloodstream, an exponential decay model is fitted to the fluorescence vs time curves to obtain a decay rate. These calculated decay rates are then used to restore the fluorescence signals to only reflect intestinal permeation. Finally, a comparison between the fluorescence signals is performed using the parameters Peak Time, Peak Intensity, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time, extracted this time from the restored fluorescence vs time curves.

To correct for the elimination of fluorescein, two different methods are investigated: *1-term* and *2-term* exponential decay fittings. The first method (*1-term*) is based on the single exponential decay rate, described by the following equation:

$$I_t = I_0 * e^{b*t} \quad (4.1)$$

, where I_t denoted the fluorescence intensity, I_0 represents the original fluorescence intensity (without the influence of fluorescein elimination), t is the time (min; starting at the time at which the first peak is reached: Peak Time value from Section 4.2.3.1: Method 3) and b is the decay rate (or the rate at which the fluorescein is metabolised/eliminated from the plasma). The *1-term* exponential decay function is fitted to the fluorescence signals, using MATLAB, to extract the decay rate (parameter b). This parameter is then used to correct the fluorescence signals, following the equation:

$$I_{corrected} = \frac{I_t}{e^{b*t}} \quad (4.2)$$

, where I_t is the measured fluorescence intensity and $I_{corrected}$ is the corrected fluorescence intensity. After the correction, the parameters Peak Intensity, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time are extracted from the restored fluorescence signals. Finally, these parameters are used to compare the data from the fluorescein-only and fluorescein + sugar studies (as in Section 4.2.3.1).

The second method (*2-term* exponential decay fitting) describes two independent decay processes that captures more realistically the complex elimination of fluorescein through the liver and kidneys [222]. It, therefore, follows the same approach as the first method (*1-term*), but using a second-order exponential decay model described by the equation:

$$I_t = a * e^{b*t} + c * e^{d*t} \quad (4.3)$$

, where I_t is the fluorescence intensity, b and d represent the decay rates and a and c represent the contribution (percentages) of the two different elimination rates to the overall clearance of the fluorescein. As in *1-term* model, the Peak Time values from Section 4.2.3.1 (Method 3) are used as the starting points for the *2-term* exponential decay model. This equation can also be expressed

as:

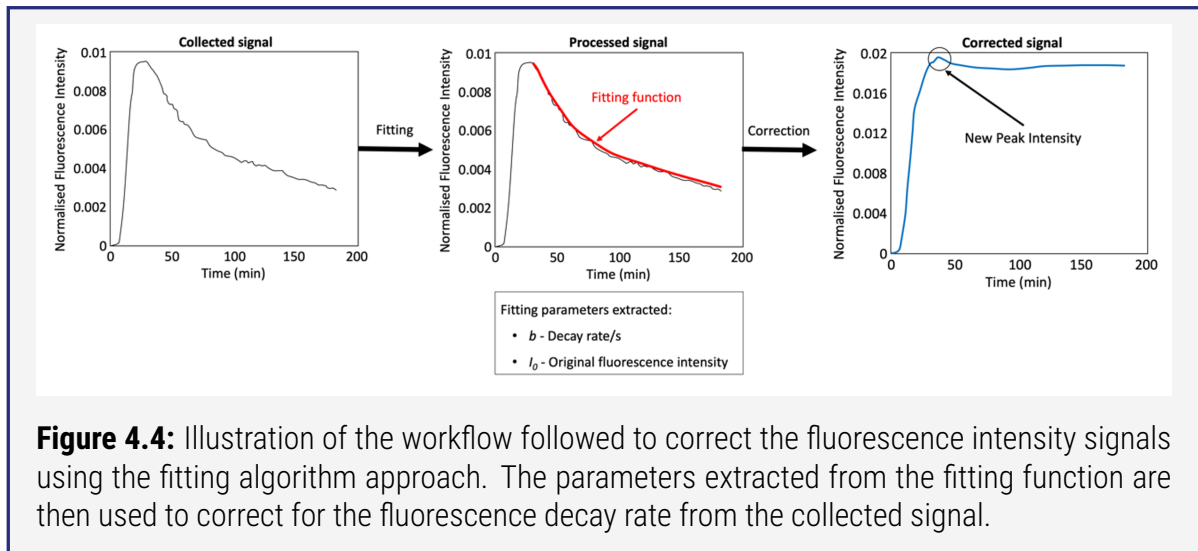
$$I_t = I_0 * \left(\frac{a}{a+c} * e^{b*t} + \frac{c}{a+c} * e^{d*t} \right) \quad (4.4)$$

, where, again, I_0 is the original fluorescence intensity (without the influence of fluorescein elimination).

Similar to the previous *1-term* approach, the decay rates b and d , extracted from the *2-term* exponential decay model are used to correct the fluorescence intensity signals, following the equation:

$$I_{corrected} = \frac{I_t}{\frac{a}{a+c} * e^{b*t} + \frac{c}{a+c} * e^{d*t}} \quad (4.5)$$

, where $I_{corrected}$ is again, the corrected fluorescence intensity. Finally, parameters extracted from the restored fluorescence signals are used to assess the level of discrimination between the datasets. An example of the process carried out for the fitting and correction of the fluorescence signals is illustrated in Fig. 4.4.



4.2.3.3 Phasor analysis

Traditional time domain analysis of fluorescence data such as the ones presented in previous sections 4.2.3.1 (automatic peak selection) and 4.2.3.2 (exponential decay fitting), are highly dependent on the design and inputs of the algorithm. For instance, the results from the peak selection and exponential decay rate algorithms are highly dependent on the values of the Peak Time and the start time of the decay components, respectively. This dependency can misrepresent other features within the data attributed to the complexity of the physiological processes captured in the signals. In this section, we focus on the frequency domain of the signals by analysing the phasor

components of the fluorescence vs time curves. Drawing from its effectiveness in previous fluorescence lifetime studies [223, 224], this approach aims to provide a two-dimensional representation of the fluorescence vs time curves, with the potential to improve the discrimination between the datasets.

The phasor analysis uses the Fourier transform to map the fluorescence curve into the frequency domain by calculating the real (g) and imaginary (s) component of the signal. To do so, it uses the following equations:

$$g(w) = \int_0^{180} I(t) * \cos(wt) dx \quad (4.6)$$

$$s(w) = \int_0^{180} I(t) * \sin(wt) dx \quad (4.7)$$

, where $I(t)$ is the fluorescence intensity, t represents the extension of the data collection (180 minutes) and w is the frequency described as: $w = 2\pi * \frac{1}{180min}$. These equations are derived from the following process:

$$\text{Fourier Transform of a signal} = F(w) \quad (4.8)$$

$$F(w) = \int_{-\inf}^{\inf} f(t) * e^{(-iwt)} dx = \int_{-\inf}^{\inf} f(t) * [\cos(wt) - i * \sin(wt)] dx \quad (4.9)$$

$$e^{(-iwt)} = \cos(wt) - i * \sin(wt); i = \text{imaginary number} \quad (4.10)$$

The values (real (g) and imaginary (s)) extracted from each signal are used in the following section 4.3.2.4 to assess the effectiveness of the phasor analysis to discriminated between both studies (fluorescein-only and fluorescein + sugar).

4.2.4 Statistical analysis - comparison between induced permeability stages

The parameters Peak AUC, Peak AUC/Slope, Peak AUC *Peak Time and Peak Intensity extracted from the approaches presented in Section 4.2.3.1(automatic peak selection) and Section 4.2.3.2 (exponential decay fitting) were used to compare the two datasets to assess which exhibited the clearest changes in response to induced changes in permeability. This comparison was performed using scatter-box plots (showing the values observed for each individual participant for both the fluorescein only and fluorescein + sugar experiments) to visualise the distribution on an individual

level and across the cohort as a whole (see section 4.3.2). In each case, statistical significance was quantified using the paired Student's T-test.

Box plots showed the median values, 25th and 75th percentiles, most extreme data points excluding outliers, and outliers for the fluorescein only and fluorescein + sugar measurements. Outliers were defined as points that fell below the 25th percentile or above the 75th percentile by more than 1.5 times the interquartile range and were plotted for visualisation purposes only. All calculations (e.g. of mean values, median values, etc.) and all statistical tests were performed based on the data points from all 11 participants (i.e. including outliers) in all cases.

For the phasor analysis, paired Student's T-test were also used to quantify the statistical significance between the studies. In addition, the phasor components were also integrated into a machine learning algorithm to assess their capability to differentiate the two permeability stages. To do so, a preliminary support vector machine (SVM) classifier was developed to differentiate between the two datasets, utilizing the phasor components from the fluorescence signals as inputs. SVM was chosen for its ability to capture intricate relationships within datasets by optimizing hyperplanes to maximize separation between data points [225]. The (SVM) classifier was developed in MATLAB and the performance metrics - validation accuracy and the parameters calculated using the confusion matrix - were used to analyse the performance of the algorithm. Further information is provided in section 4.3.2.4.

4.3 Results

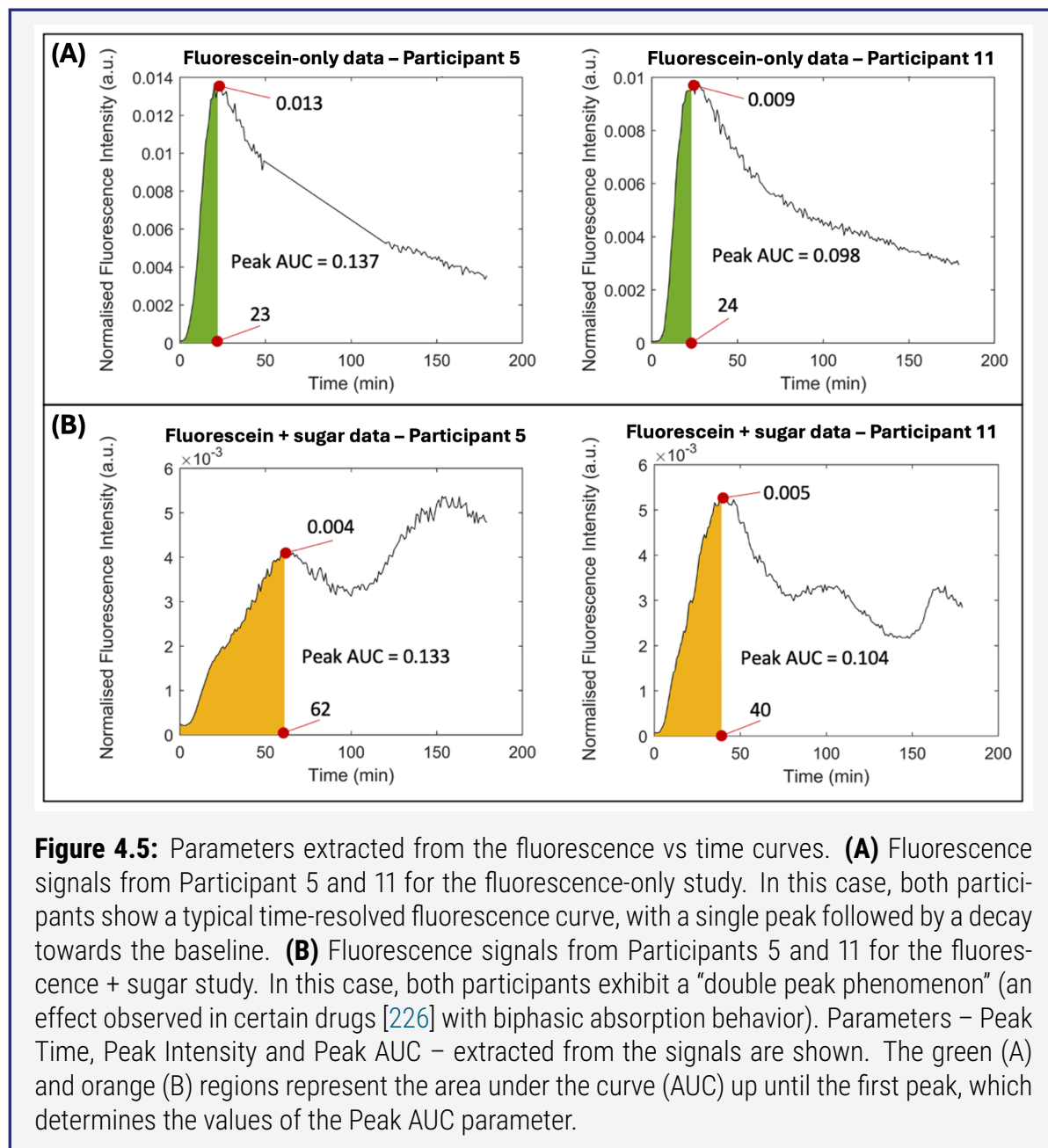
4.3.1 Signal processing and computational parameter extraction

4.3.1.1 Automatic Peak Selection

As explained in Section 4.2.3.1, the first analysis of the fluorescence vs time curves focuses on the investigation of three methods for the identification of the Peak time, providing different degrees of automation.

The first method (*Method 1*) involves the manual extraction of the parameters/features – Peak Time, Peak Intensity, Slope and Peak AUC – from the fluorescence vs time signals. To do so, the fluorescence vs time curves were uploaded into an excel file and manually inspected by the clinician Dr Jonathan Gan. The first peak in the fluorescence vs time curves was identified and the Peak Time (*x value*) and Peak Intensity (*y value*) were manually extracted. The Slope (average gradient of the first permeation stage of the curve) and the Peak AUC parameters were calculated computationally in MATLAB. The Peak AUC values used the trapezoidal numerical integration (*trapz* MATLAB function) method that calculates the area under the curve (AUC) of the signal up to the Peak Time by dividing the area of this region into smaller trapezoids. It, therefore, approximates this region under the graph as a sum of trapezoidal areas. Figure 4.5 shows the Peak Time, Peak

Intensity and Peak [AUC](#) parameters on two examples of fluorescence vs time curves from both experimental conditions (Fluorescein and Fluorescein + sugar).



The Peak [AUC](#) values were then corrected for differences in gastric emptying rate (as mentioned in Section 4.2.3.1) by either dividing by Slope parameter (i.e., the section of the curve up until the first peak) or by multiplying by the time at which the first peak was observed (Peak Time). This correction was required as a later peak (which indicates slower gastric emptying) means that more fluorescein would have been eliminated from the body by the time the peak had been reached, thus reducing the [AUC](#) value. The resulting parameters (Peak Time, Peak Intensity, Peak [AUC](#), Peak [AUC](#)/slope, and Peak [AUC](#)*time) for each participant and experimental condition are displayed in Table 4.2. Later Section 4.3.2.2 will then use these parameters to compare between the

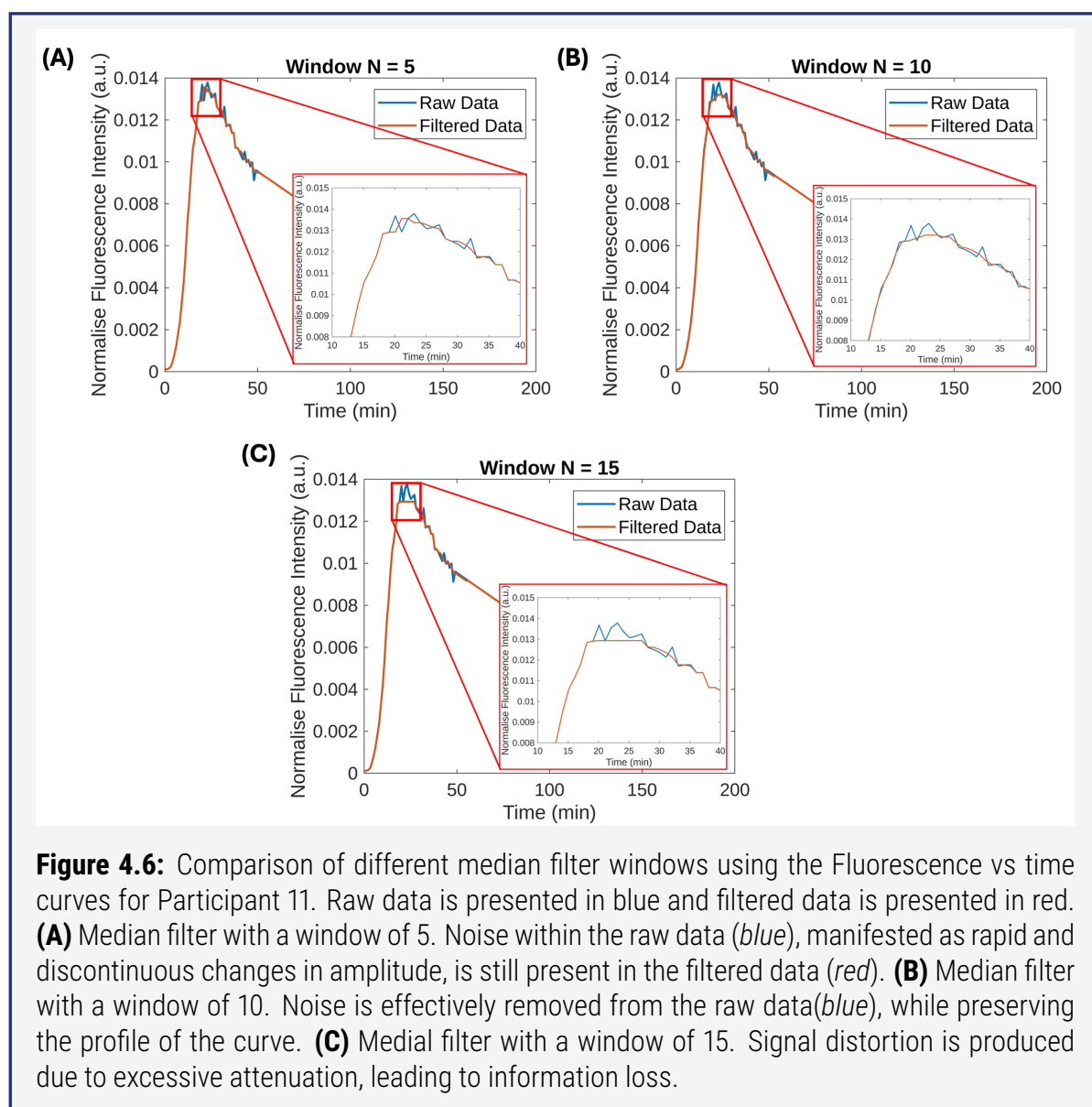
two permeability stages and to determine the potential of each calculated/extracted parameter to discriminate between datasets.

Table 4.2: Parameters extracted from the fluorescence vs time curves for all 11 participants using Method 1. Peak Time values were manually extracted from the raw signals. The rest of the parameters were calculated computationally in MATLAB. F - Fluorescence only; F + s - Fluorescence + sugar.

ID	Test	Peak Time	Peak Intensity	Peak AUC	Slope	Peak AUC/ Slope	Peak AUC* Peak Time
P1	F	23	0.0043	0.042	1.910×10^{-4}	219	0.966
	F + s	40	0.0043	0.086	1.080×10^{-4}	795	3.443
P2	F	64	0.0013	0.033	1.900×10^{-5}	1666	2.119
	F + s	40	0.0041	0.092	1.020×10^{-4}	899	3.694
P3	F	24	0.0074	0.077	3.230×10^{-4}	239	1.852
	F + s	74	0.0032	0.118	4.300×10^{-5}	2728	8.755
P4	F	31	0.0048	0.076	1.600×10^{-4}	472	2.355
	F+s	49	0.0014	0.046	1.800×10^{-5}	2459	2.264
P5	F	23	0.0135	0.137	6.150×10^{-4}	223	3.165
	F+s	62	0.0041	0.133	6.300×10^{-5}	2111	8.295
P6	F	40	0.0017	0.031	4.200×10^{-5}	713	1.218
	F+s	32	0.0044	0.069	1.319×10^{-4}	526	2.22
P7	F	22	0.0063	0.049	3.010×10^{-4}	164	1.087
	F+s	40	0.002	0.057	4.500×10^{-5}	1272	2.303
P8	F	40	0.0015	0.019	3.700×10^{-5}	526	0.779
	F+s	32	0.0026	0.046	8.030×10^{-5}	580	1.49
P9	F	27	0.0043	0.057	1.620×10^{-4}	354	1.551
	F+s	58	0.0025	0.1	4.200×10^{-5}	2389.8	5.824
P10	F	20	0.0055	0.026	2.860×10^{-4}	92	0.53
	F+s	40	0.0034	0.067	8.500×10^{-5}	788	2.692
P11	F	24	0.0095	0.098	4.150×10^{-4}	237	2.363
	F+s	40	0.0052	0.104	1.320×10^{-4}	785	4.164
Mean	F	30.727	0.0055	0.059	2.319×10^{-4}	445.909	1.635
	F+s	46.091	0.0034	0.083	7.729×10^{-5}	1393.891	4.104
STD	F	13.016	0.0037	0.036	1.809×10^{-4}	443.605	0.81
	F+s	13.285	0.0012	0.029	3.845×10^{-5}	848.076	2.485

The second method (*Method 2*) implemented a filtering step to reduce the noise within the fluo-

rescence vs time signals. Upon inspecting the dataset, it was observed that the noise within the fluorescence vs time curves primarily consisted of impulse noise or occasional outliers. These outliers could be attributed to movements of the optical probe exposing the detector to ambient light and causing sudden spikes/increases in the signal. Using a median filter proved to be effective in removing these occasional outliers as it replaces the centre value in the sample window with the median of the values within that window. Different sample windows widths were investigated. Figure 4.6 displays a fluorescence vs time curve from participant 5 with a median filtering algorithm with three different window widths ($N = 5, 10, 15$) investigated. As we can see from the zoom in graphs within Figure 4.6, window widths < 10 does not remove accurately all the outliers, whereas widths > 10 results in excessive attenuation, which manifests as flattened regions within the signal. Therefore, a median filter with a window width = 10 was selected as proved to efficiently remove the outliers without significantly affecting the signal.



Following with *Method 2*, the Peak Times were once again determined manually by Dr Jonathan Gan, but this time using the filtered data. For Peak AUC values, manually selected Peak Times (this time from the filter signals) were used for the calculation. As in *Method 1*, Slope was also calculated computationally in MATLAB. The resulting parameters, including the corrected Peak AUC, (Peak Time, Peak Intensity, Peak AUC, AUC/slope, and AUC*time) are displayed in Table 4.3. As mentioned before, these parameters will be then used in Section 4.3.2.2 to compare between the two permeability stages.

Last method investigated for the Peak Time selection (*Method 3*) is the most automatic approach. As in *Method 2*, Fluorescence vs time curves are filtered using the median filter function in MATLAB, *medfilt1* (width N= 10). For the automatic detection of the first peak in the signals and therefore, the Peak times, the MATLAB function “*findpeaks*” is used. This function, based on the derivative of the input signal, returns the local maxima (local peak) of the signal. This means that if a sample is larger than its two neighboring samples, it is labelled as a peak. Because of how the function calculates the local maxima, multiple peaks are simultaneously detected on each signal. A considerable proportion of these peaks stemmed from residual noise after the filtering process and do not accurately represent the first peak within the fluorescence signal relevant to the permeation of the fluorescein. To remove these noise artifacts and to extract exclusively the first peak of the signal, the full width at half-maximum (FWHM), also extracted from the MATLAB function (*findpeaks*), was used as a threshold parameter. This parameter (FWHM) reflects the temporal duration of the peak and therefore, can be used to ignore peaks resulted from remanence noise, characterized by very short durations. Following these considerations, peaks with a duration (FWHM) greater than 4 minutes were considered as valid peaks. From this subset, peaks occurring at earliest times (first peak) were selected as Peak Times. In cases where fluorescence signals, such as those from Participants 2 and 6, do not show clear peaks within the expected times (< 60 minutes) values detected as Peak Times underwent expert consultation.

Once the Peak times and Peak intensity values were extracted computationally, the Peak AUC was calculated in MATLAB using the trapezoidal numerical integration, as described in *Method 1* and 2. Slope was also calculated in MATLAB and Peak AUC values were corrected accordingly, using the Peak Time and Slope values. Finally, the resulting parameters (Peak Time, Peak Intensity, Peak AUC, AUC/slope, and AUC*time) were used to compare between the datasets (see Section 4.3.2.2). Table 4.4 shows the values for each feature/parameter extracted/calculated for each signal.

Table 4.3: Parameters extracted from the fluorescence vs time curves for all 11 participants using Method 2. Peak Time were manually extracted from the filtered signals. The rest of the parameters were calculated computationally in MATLAB. F - Fluorescence only; F + s - Fluorescence + sugar.

ID	Test	Peak Time	Peak Intensity	Peak AUC	Slope	Peak AUC/ Slope	Peak AUC* Peak Time
P1	F	23	0.0042	0.039	1.911×10^{-4}	205.619	0.904
	F+s	40	0.0043	0.083	1.114×10^{-4}	748.092	3.332
P2	F	64	0.0013	0.032	1.994×10^{-5}	1614.926	2.061
	F+s	40	0.004	0.089	1.027×10^{-4}	872.444	3.584
P3	F	24	0.0074	0.0725	3.251×10^{-4}	222.981	1.74
	F+s	74	0.0032	0.116	4.304×10^{-5}	2699.814	8.599
P4	F	31	0.0048	0.073	1.591×10^{-4}	458.946	2.263
	F+s	49	0.0014	0.045	1.719×10^{-5}	1669.977	2.225
P5	F	23	0.0131	0.128	5.976×10^{-4}	215.682	2.965
	F+s	62	0.0041	0.131	6.564×10^{-5}	1998.903	8.134
P6	F	40	0.0019	0.029	4.833×10^{-5}	604.23	1.168
	F+s	32	0.0042	0.066	1349×10^{-4}	495.218	2.138
P7	F	22	0.0054	0.045	2.603×10^{-4}	174.428	0.999
	F+s	40	0.002	0.056	4.917×10^{-5}	1147.111	2.256
P8	F	40	0.0012	0.018	3.169×10^{-5}	580.606	0.736
	F+s	32	0.0026	0.044	8.292×10^{-5}	541.492	1.437
P9	F	27	0.0042	0.054	1.631×10^{-4}	335.357	1.477
	F+s	58	0.0025	0.098	4.355×10^{-5}	2264.272	5.719
P10	F	20	0.0046	0.023	2.449×10^{-4}	96.366	0.472
	F+s	40	0.0033	0.065	8.374×10^{-5}	776.194	2.6
P11	F	24	0.0095	0.092	4.150×10^{-4}	223.154	2.222
	F+s	40	0.0052	0.101	1.326×10^{-4}	760.41	4.032
Mean	F	30.727	0.0052	0.055	2.323×10^{-4}	430.208	1.546
	F+s	46.091	0.0033	0.081	7.970×10^{-5}	1270.357	4.005
STD	F	13.016	0.0036	0.033	1.751×10^{-4}	427.251	0.771
	F+s	13.284	0.0011	0.028	3.733×10^{-5}	761.859	2.449

Table 4.4: Parameters extracted from the fluorescence vs time curves using Method 3. All Peak Time values generated by the computational algorithm in Method 3 were validated with a clinician. Only, Participant 2 and 6 showed unclear Peak Time values (146 and 83, respectively). Therefore, new Peak Time values were suggested and are included in brackets (71 and 48, respectively). For these two participants, the rest of the parameters were calculated using both Peak Time values. However, only values from within the brackets are considered for the analysis. The mean and STD are calculated using all individual values from each participant, and only the suggested values (in brackets) for participants 2 and 6 are considered. F - Fluorescence only; F + s - Fluorescence + sugar.

ID	Test	Peak Time	Peak Intensity	Peak AUC	Slope	Peak AUC/ Slope	Peak AUC* Peak Time
P1	F	27	0.0042	0.056	$1.610 \cdot 10^{-4}$	348.361	1.515
	F + s	45	0.0044	0.105	$9.895 \cdot 10^{-5}$	1061.131	4.725
P2	F	146 (71)	0.0024 (0.0013)	0.177 (0.0413)	$1.619 \cdot 10^{-5}$ ($1.851 \cdot 10^{-5}$)	10954.588 (2231.219)	25.886 (2.933)
	F + s	45	0.0040	0.110	$9.151 \cdot 10^{-5}$	1199.843	4.941
P3	F	31	0.0076	0.125	$2.528 \cdot 10^{-4}$	495.966	3.887
	F + s	67	0.0032	0.094	$4.782 \cdot 10^{-5}$	1965.828	6.298
P4	F	37	0.0048	0.102	$1.322 \cdot 10^{-4}$	769.929	3.767
	F + s	53	0.0014	0.051	$2.53 \cdot 10^{-5}$	2013.025	2.703
P5	F	27	0.0131	0.182	$5.058 \cdot 10^{-4}$	359.028	4.903
	F + s	66	0.0041	0.148	$6.170 \cdot 10^{-5}$	2390.755	9.735
P6	F	83 (48)	0.0025 (0.0019)	0.126 (0.0447)	$3.025 \cdot 10^{-5}$ ($4.114 \cdot 10^{-5}$)	4168.457 (1086.486)	10.466 (2.144)
	F + s	48	0.0053	0.14	$1.111 \cdot 10^{-4}$	1262.267	6.73
P7	F	23	0.0054	0.051	$2.483 \cdot 10^{-4}$	204.558	1.168
	F + s	52	0.0019	0.079	$3.807 \cdot 10^{-5}$	2075.125	4.108
P8	F	45	0.0013	0.025	$2.908 \cdot 10^{-5}$	852.732	1.116
	F + s	40	0.0026	0.066	$6.715 \cdot 10^{-5}$	979.925	2.632
P9	F	31	0.0042	0.072	$1.414 \cdot 10^{-4}$	507.108	2.223
	F + s	39	0.0023	0.055	$6.005 \cdot 10^{-5}$	909.242	2.129
P10	F	28	0.0055	0.067	$2.051 \cdot 10^{-4}$	328.069	1.884
	F + s	46	0.0033	0.085	$7.393 \cdot 10^{-5}$	1151.104	3.915
P11	F	28	0.0095	0.131	$3.546 \cdot 10^{-4}$	368.616	3.66
	F + s	45	0.0052	0.127	$1.173 \cdot 10^{-4}$	1079.284	5.697
Mean	F	36.000	0.0053	0.081	$1.900 \cdot 10^{-4}$	686.557	2.655
	F + s	49.636	0.0034	0.096	$7.208 \cdot 10^{-5}$	1462.503	4.874
STD	F	14.000	0.0036	0.048	$1.478 \cdot 10^{-4}$	577.291	1.258
	F + s	9.341	0.0013	0.033	$2.979 \cdot 10^{-5}$	533.513	2.204

4.3.1.2 Exponential decay fitting

The use of a high viscosity solution, such as the one used in the fluorescence + sugar group, could potentially cause a decreased in the gastric emptying rate, as observed in [209]. This variations in the gastric emptying rate are directly linked to a lower fluorescence intensity detected due to the continuing elimination of the fluorescein within the bloodstream. The second analysis of the fluorescence vs time curves focuses, then, on investigating two decay-rate fitting approaches to correct for the impact that the clearance phase (i.e., decay rate) has on the fluorescence signals with the ultimate goal being to improve the discrimination between gut permeability stages and to correct for the variations in the gastric emptying rate due to for example, the use of high viscosity solutions.

At the start of both fitting models (*1-term* and *2-term*), a median filter (window $N=10$) was applied to the fluorescence intensity signal to remove any noise that could impact the fitting approaches. The clearance phase (depicted in Figure 4.2) within the fluorescein signal is characterised by an exponential decay with the starting point being the Peak Time (i.e., the time at which the first peak is reached; see Figure 4.2). Therefore, in this case, Peak Time values extracted from the previous section 4.3.2.2 (*Method 3*) were used as the starting points for both decay models (*1-term* and *2-term*). Figure 4.7 shows two examples of the processes followed in the 1-term and 2-term models, respectively, to predict the exponential decay function to later correct the fluorescence signals.

For *1-term* model, the single exponential decay function presented in Equation 4.1 was fitted to the clearance region of the fluorescence signals using the MATLAB function "*fitytype*" and "*fitoptions('exp1')*" (Figure 4.7 blue lines). The resulting parameters, I_0 (original fluorescence intensity) and b (decay rate) were extracted for each participant. Table 4.5 shows the predicted I_0 and b values, as well as the R^2 and RMSE used to evaluate the accuracy of the prediction for *1-term* model.

As observed in Fig. 4.8, some of the Peak Time values (from *Method 3*, Section 4.3.2.2) used for the *1-term* fitting model do not represent a feasible starting point for the fitting algorithms, as the subsequent data points do not have a clear exponential decay, which lead to inaccurate parameter prediction. For those cases, Peak Time values were substituted for new starting points (previously validated by an expert) and I_0 and b , R^2 and RMSE values were recalculated. Participant 2, however, was excluded from the analysis due to the lack of an exponential decay region (clearance phase) for the fitting model to work with and the potential incorrect prediction of the parameters I_0 and b . Table 4.5 shows the new starting points, as well as recalculated values I_0 and b , R^2 and RMSE in brackets (values for participant 2 are shown, but are not considered for future analysis).

In addition to the new values presented in Table 4.5, the new fitting curves for the participants (Participants 5, 6 and 8) can also be seen in Figure 4.9. Overall, these adjustments have improved the fitting and the predicted values of the datasets, with mean R^2 values of 0.946 and 0.765 for

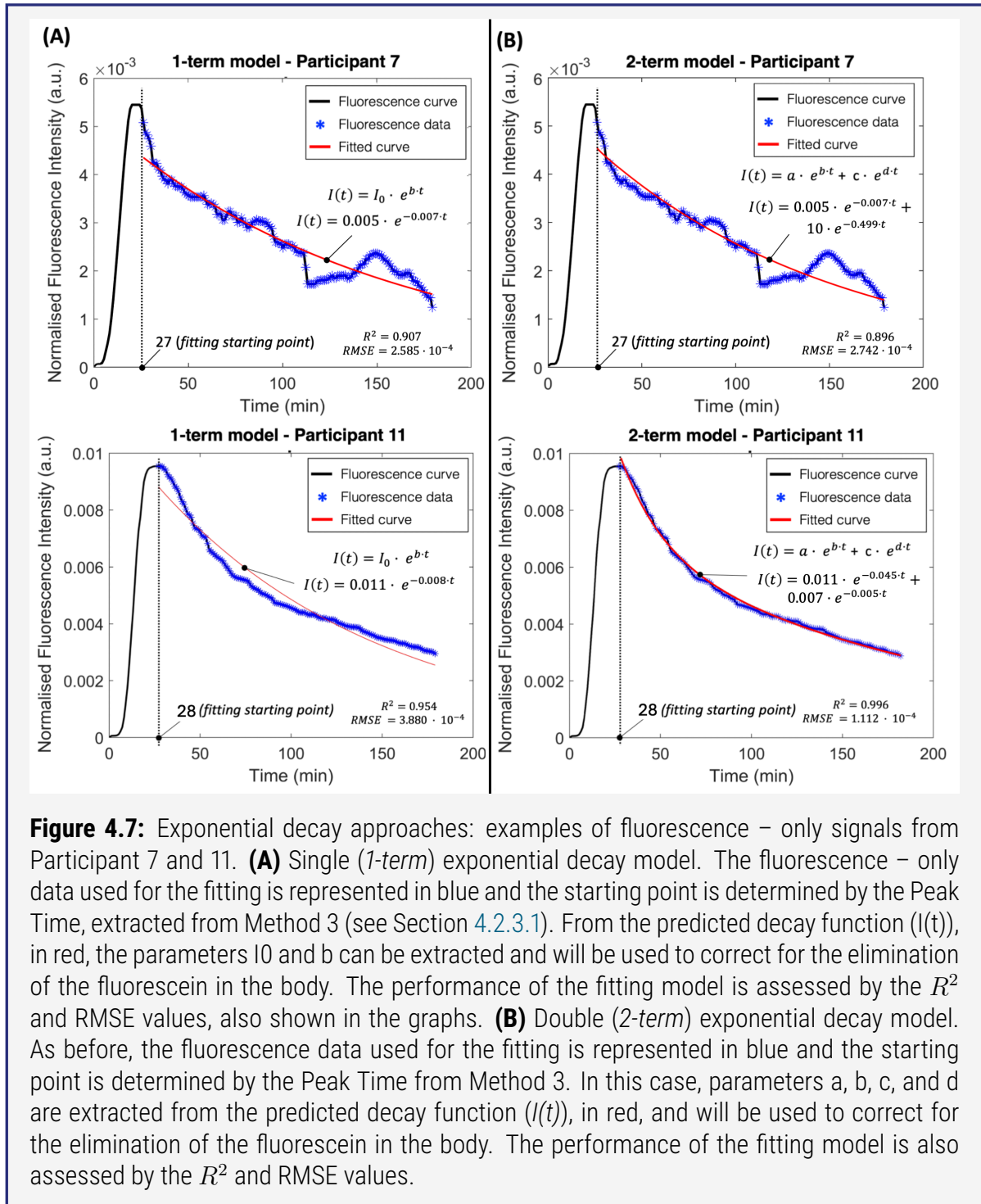
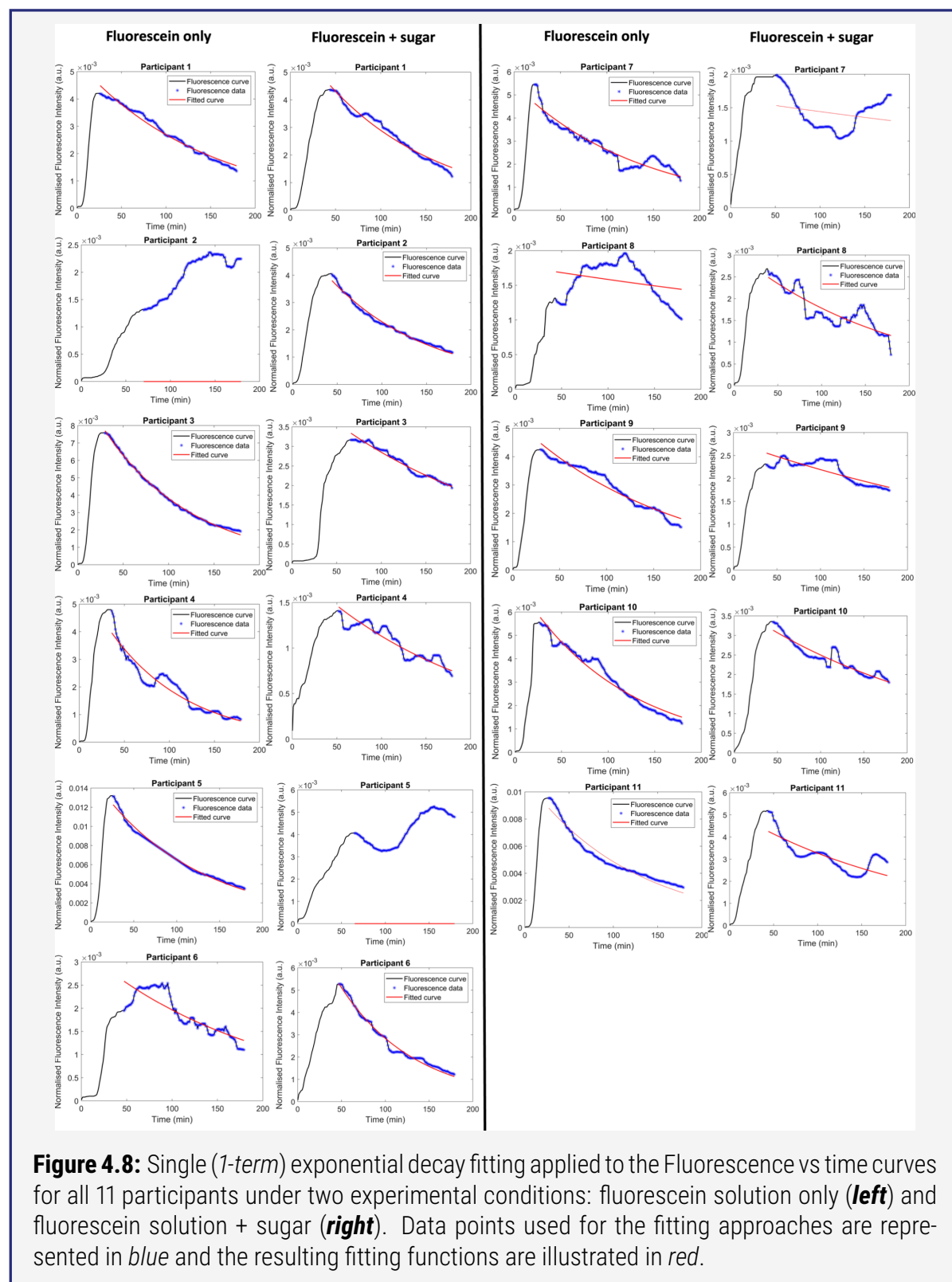
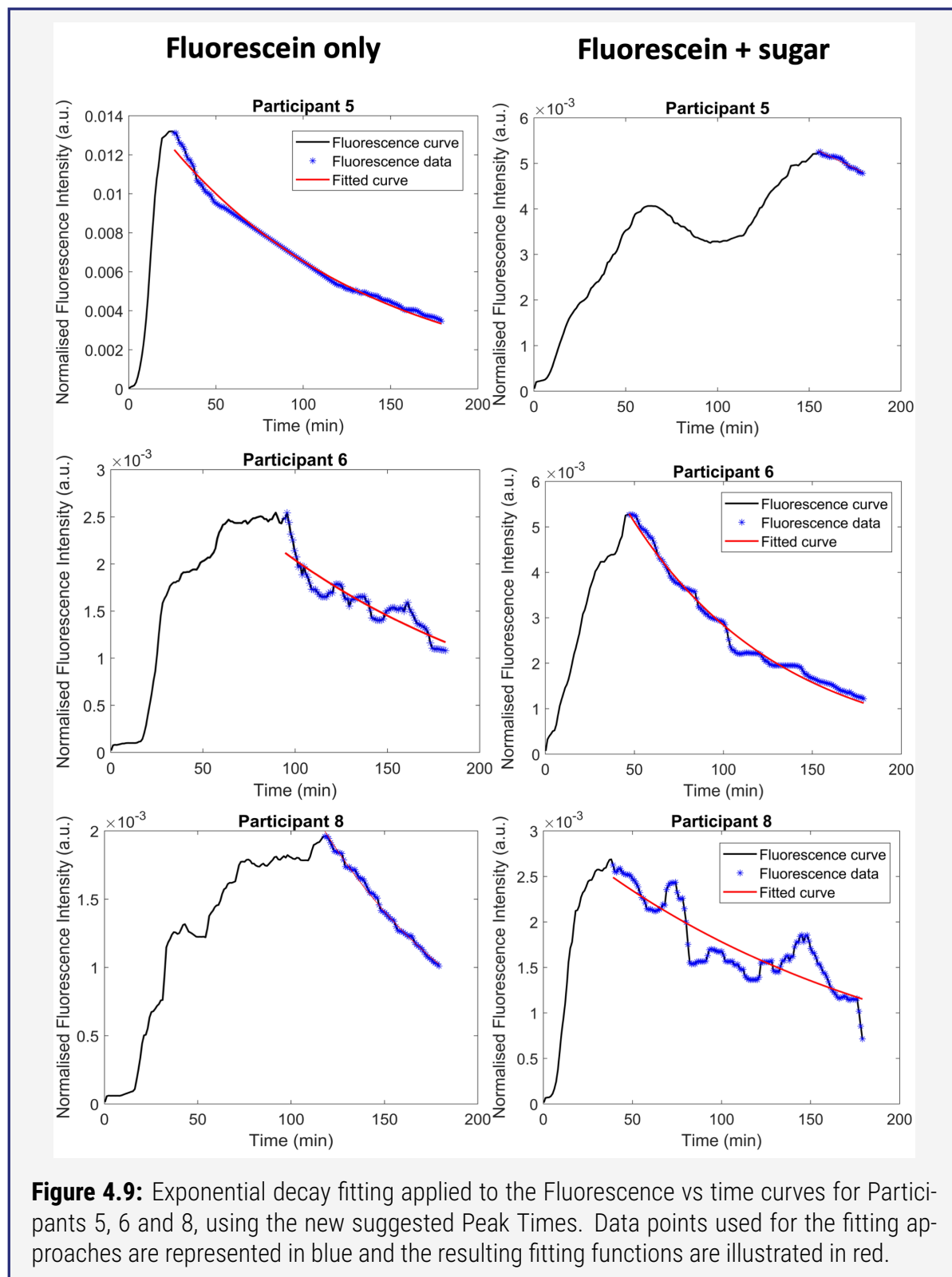


Table 4.5: Predicted parameters extracted from the single (1-term) exponential fitting curve. The Peak Time values, from Method 3 (see Section 4.3.1.1) The predicted parameters I_0 and b are used to correct the fluorescence signal for the elimination of fluorescein from the body. The performance of the fitting is assessed by the R^2 and RMSE values, also shown. Values from Participant 2 are only displayed for visual information, and they are not used for the analysis nor the calculation of the mean and STD values.

ID	Test	Peak Time	I_0	b	R^2	RMSE
P1	F	27	$5.380 \cdot 10^{-3}$	-0.0069	0.979	$1.240 \cdot 10^{-4}$
	F+s	45	$6.419 \cdot 10^{-3}$	-0.008	0.979	$1.542 \cdot 10^{-4}$
P2	F	71	$9.413 \cdot 10^{-4}$	-0.5	-28.291	$2.000 \cdot 10^{-3}$
	F+s	45	$5.662 \cdot 10^{-3}$	-0.009	0.988	$8.639 \cdot 10^{-5}$
P3	F	31	$1.041 \cdot 10^{-2}$	-0.0101	0.998	$8.228 \cdot 10^{-5}$
	F+s	67	$4.538 \cdot 10^{-3}$	-0.0047	0.971	$6.951 \cdot 10^{-5}$
P4	F	37	$5.990 \cdot 10^{-3}$	-0.0115	0.925	$2.581 \cdot 10^{-4}$
	F+s	53	$1.903 \cdot 10^{-3}$	-0.0052	0.829	$7.984 \cdot 10^{-5}$
P5	F	27	$1.530 \cdot 10^{-2}$	-0.0085	0.991	$2.486 \cdot 10^{-4}$
	F+s	66 (156)	$2.112 \cdot 10^{-3}$ $(9.637 \cdot 10^{-3})$	-0.5 (-0.0039)	-34.114 (-0.942)	$4.300 \cdot 10^{-3}$ $(3.694 \cdot 10^{-5})$
P6	F	48 (95)	$3.298 \cdot 10^{-3}$ $(4.028 \cdot 10^{-3})$	-0.0052 (-0.0068)	0.737 (0.813)	$2.285 \cdot 10^{-4}$ $(1.311 \cdot 10^{-4})$
	F+s	48	$9.191 \cdot 10^{-3}$	-0.0118	0.987	$1.386 \cdot 10^{-4}$
P7	F	27	$5.253 \cdot 10^{-3}$	-0.0069	0.907	$2.585 \cdot 10^{-4}$
	F+s	52	$1.653 \cdot 10^{-3}$	-0.0012	0.514	$2.539 \cdot 10^{-4}$
P8	F	45 (119)	$1.787 \cdot 10^{-3}$ $(7.265 \cdot 10^{-3})$	-0.0012 (-0.0109)	0.088 0.995	$2.598 \cdot 10^{-4}$ $(2.124 \cdot 10^{-5})$
	F+s	40	$3.086 \cdot 10^{-3}$	-0.0055	0.763	$2.147 \cdot 10^{-4}$
P9	F	31	$5.357 \cdot 10^{-3}$	-0.0059	0.945	$1.916 \cdot 10^{-4}$
	F+s	39	$2.802 \cdot 10^{-3}$	-0.0025	0.649	$1.472 \cdot 10^{-4}$
P10	F	28	$7.336 \cdot 10^{-3}$	-0.0089	0.962	$2.513 \cdot 10^{-4}$
	F+s	46	$3.796 \cdot 10^{-3}$	-0.0041	0.883	$1.402 \cdot 10^{-4}$
P11	F	28	$1.100 \cdot 10^{-2}$	-0.0082	0.954	$3.878 \cdot 10^{-4}$
	F+s	45	$5.232 \cdot 10^{-3}$	-0.0047	0.604	$4.491 \cdot 10^{-4}$
Mean	F	44.6	$7.729 \cdot 10^{-3}$	-0.0085	0.946	$1.953 \cdot 10^{-4}$
	F+s	59.1	$4.839 \cdot 10^{-3}$	-0.0052	0.765	$1.693 \cdot 10^{-4}$
STD	F	33.567	$3.490 \cdot 10^{-3}$	0.0018	0.056	$1.071 \cdot 10^{-4}$
	F+s	34.962	$2.842 \cdot 10^{-3}$	0.0029	0.285	$1.247 \cdot 10^{-4}$



both fluorescence-only and fluorescence + sugar studies (note that participant 2 was excluded from this calculation).



When a double exponential decay (2-term) model is applied to the same data, some of the participants exhibited higher R^2 values attributed to an improved estimation of the clearance region

compared with the previous 1-term model (Figure 4.7). However, in some cases (for example, fluorescein + sugar signal from Participants 6 and 7), the fittings exhibited lower R^2 values. Nevertheless, the overall dataset does not show a considerable improvement, with mean R^2 of 0.952 (fluorescein -only) and 0.741 (fluorescein + sugar). Table 4.6 shows the parameters calculated for each participants using 2-term model.

Table 4.6: Predicted parameters extracted from the double (2-term) exponential fitting curve. The Peak Time values, from Method 3 (see Section 4.3.1.1) determine the starting point of the fitting curve. The predicted parameters a , b , c and d are used to correct the fluorescence signal for the elimination of fluorescein from the body. The performance of the fitting is assessed by the R^2 , also shown.

ID	Test	Peak Time	a	c	b	d	R^2
P1	F	27	0.0054	5.104×10^{-1}	-0.007	-11.575	0.979
	F+s	45	0.0076	5.140×10^{-1}	-0.009	-10.353	0.928
P3	F	31	0.0104	1.451×10^{-8}	-0.010	-0.500	0.998
	F+s	67	0.0029	1.700×10^{-3}	-0.005	-0.005	0.971
P4	F	37	0.006	9.006×10^{-5}	-0.012	-0.019	0.925
	F+s	53	0.0019	5.104×10^{-1}	-0.005	-0.197	0.801
P5	F	27	0.0154	10.000×10^0	-0.009	-0.499	0.991
	F+s	156	0.0121	5.000×10^{-1}	-0.005	-0.196	0.832
P6	F	95	0.0299	3.500×10^{-3}	-0.052	-0.006	0.834
	F+s	48	0.0082	5.390×10^{-3}	-0.031	-0.008	0.992
P7	F	27	0.0055	10.000×10^0	-0.007	-0.499	0.896
	F+s	52	0.0018	3.949×10^{-5}	-0.002	-0.500	0.068
P8	F	120	0.0078	4.964×10^{-1}	-0.011	-0.084	0.981
	F+s	40	0.0062	2.167×10^{-3}	-0.050	-0.003	0.776
P9	F	31	0.0055	5.204×10^{-1}	-0.006	-10.618	0.943
	F+s	39	0.0029	4.792×10^{-5}	-0.003	-0.005	0.611
P10	F	28	0.0073	5.091×10^{-1}	-0.009	-10.573	0.962
	F+s	46	0.0008	3.300×10^{-3}	-0.017	-0.003	0.894
P11	F	28	0.0113	7.700×10^{-3}	-0.045	-0.005	0.996
	F+s	45	0.0052	7.212×10^{-11}	-0.005	-0.500	0.604
Mean	F	45.7	0.0105	2.205×10^0	-0.017	-3.438	0.952
	F+s	59.1	0.0049	1.537×10^{-1}	-0.013	-1.177	0.741
STD	F	33.567	0.0075	4.115×10^0	0.002	5.176	0.053
	F+s	34.962	0.0036	2.447×10^{-1}	0.015	3.230	0.201

As previously described, fluorescein is eliminated from the body through the liver and the kidneys, which have different elimination rates [222]. Therefore, a double exponential decay model was expected to improve considerable the fitting of the signals because it can represent more accurately the heterogeneity and complexity of these elimination processes. However, for both fitting models (1-term and 2-term) to predict accurate values, the fluorescence signals need to exhibit a clear exponential decay region. This is not the case in all participants, as shown in Fig. 4.8, where part of the signal within the expected clearance region (i.e., after the first peak) has not reach a constant decay and, in some cases, the signal continues increasing (such as Participants 5 and 7). Longer collection times (>180 minutes) could provide a better estimation of the decay regions, potentially improving the prediction of the parameters. However, prolonged collection times may lead to patient discomfort and diminish signal quality due to motion artifacts or even participant's withdrawals, which could difficult data interpretation.

Therefore, following with the approaches proposed previously (1-term and 2-term exponential decay fittings), the fluorescence signals were corrected using the predicted parameters (I_0 and b for 1-term model and a, b, c and d for 2-term model). Figure 4.10 shows some examples of the corrected fluorescence signals for 1-term and 2-term models. These examples demonstrate the effect that the accuracy of the prediction has when correcting the fluorescence signals. High R^2 values, such as the examples presented in Fig. 4.10A allow for accurate prediction of the parameters (I_0 and b for 1-term model and a, b, c and d for 2-term model) and a better correction of the clearance regions. However, participants in which $R^2 < 0.900$ (Fig. 4.10)B exhibit inaccurate correction of the elimination region due to the incorrect prediction of the parameters. In addition, we have also observed that small changes to the Peak Time values have a big impact on the resulting correction of the fluorescence signals. To illustrate this phenomenon, Fig. 4.11 shows an example of Participant 5, in which the original Peak Time (27 min) was modified to 20 min, leading to a significant decrease in the maximum intensity of the resulting corrected fluorescence signal. This suggests that the proposed models are very sensitive to variations in input data which could potentially lead to unrealistic predictions and incorrect assumptions.

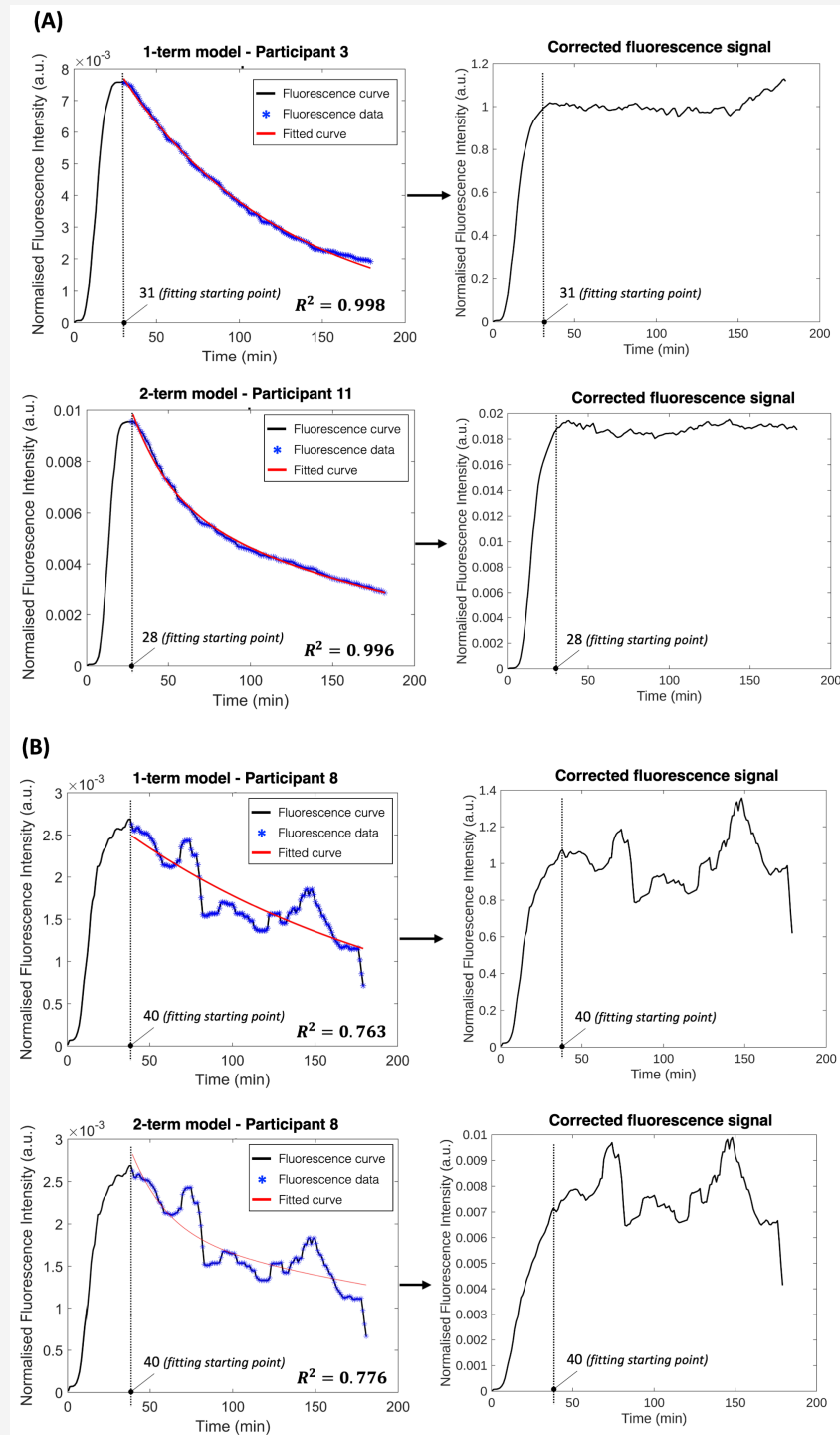
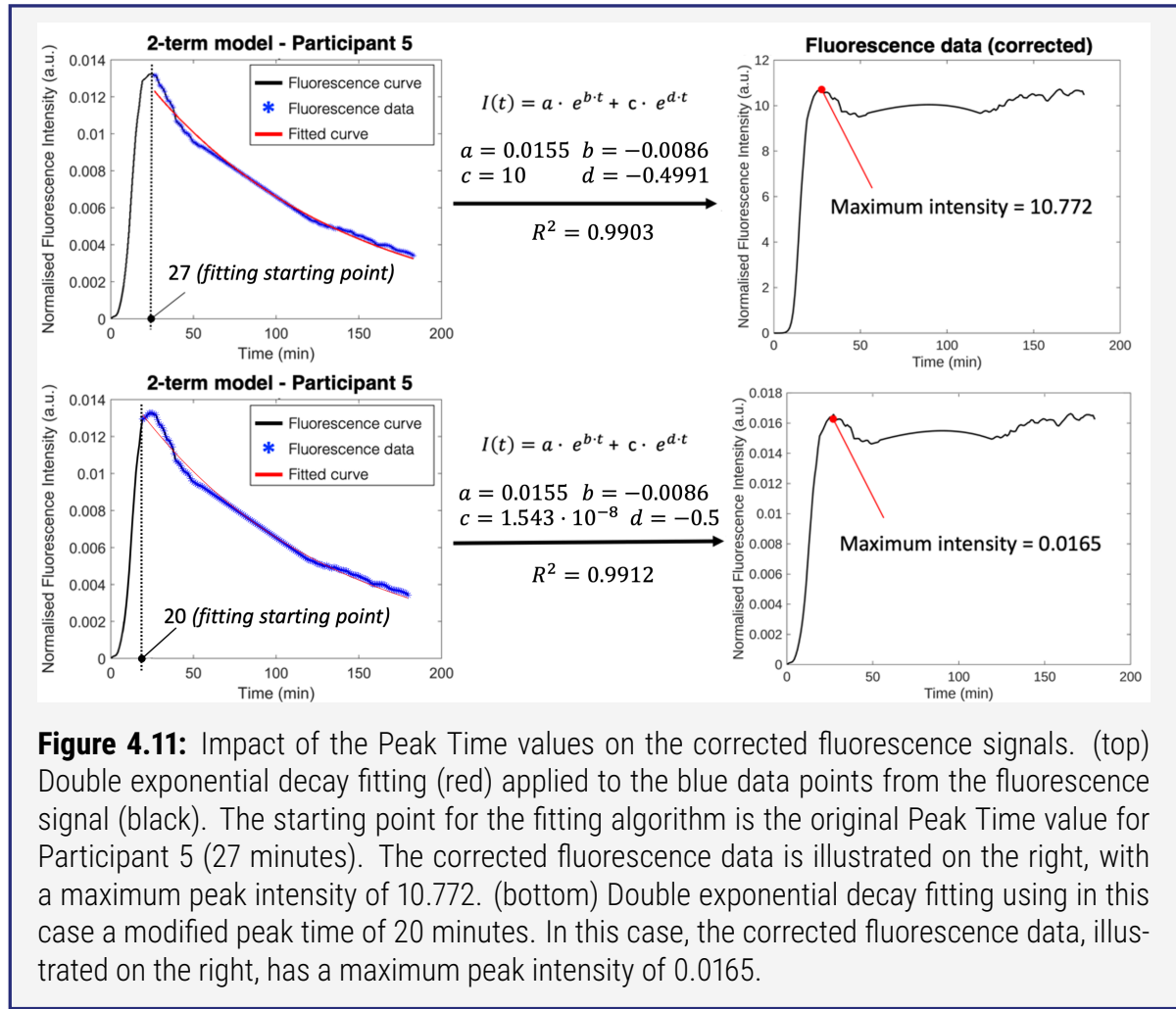
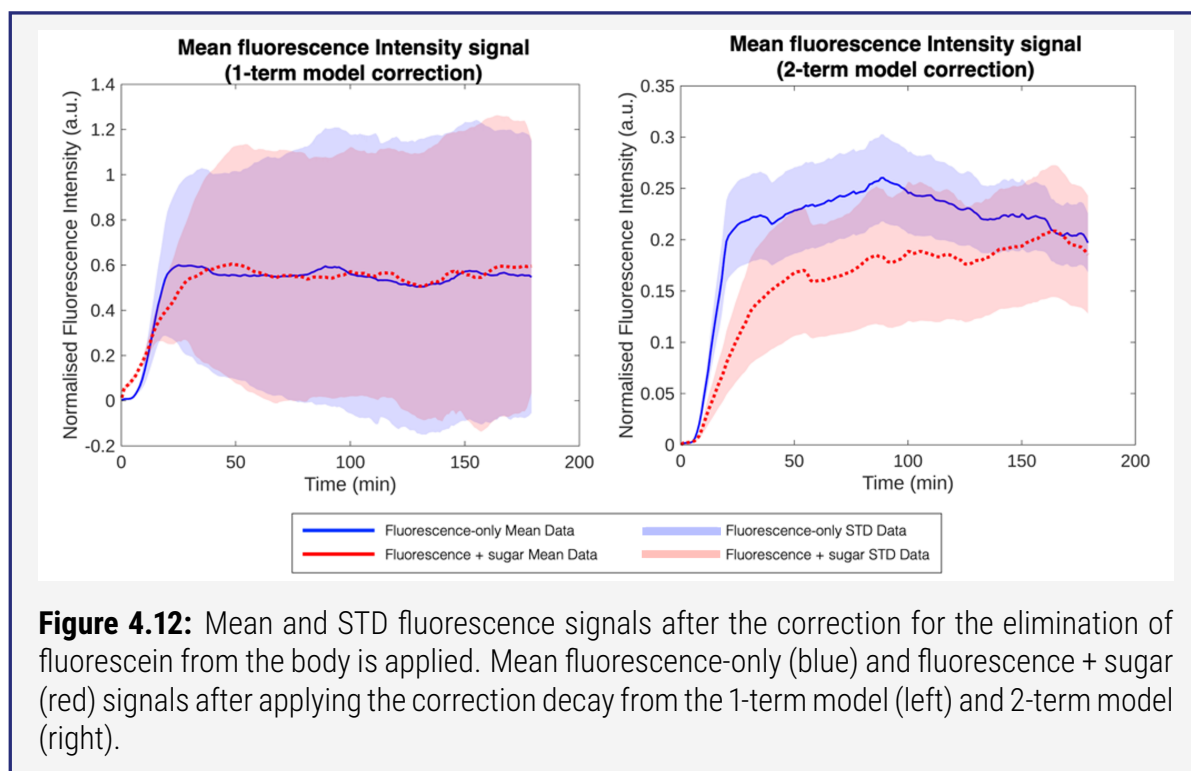


Figure 4.10: Exponential decay correction using the predicted parameters extracted from 1-term and 2-term approaches. **(A)** Fluorescence signals from Participants 3 and 11 (left graphs) and the fitting functions (in red) predicted by 1-term (*top*) and 2-term (*bottom*) approaches. In this case, both exponential decay fitting approaches provided R^2 values > 0.900 . This determined a good prediction of the clearance of fluorescein, recovering the data on the clearance phase to a plateau profile (right). **(B)** Fluorescence signals from Participants 8 (left graphs) and the fitting functions (in red) predicted by 1-term (*top*) and 2-term (*bottom*) approaches. In this case, both exponential decay fitting approaches provided R^2 values < 0.800 . This determined an inaccurate prediction of the clearance of fluorescein (see right graphs).



Continuing with the analysis, the mean fluorescence vs time curves after correction were calculated. As illustrated in Fig. 4.12, the single exponential decay model provided the expected correction of the elimination phase (i.e., resulting in a plateau region after the Peak Time). However, no differences are observed between the datasets when using the single exponential decay model (1-term). It is only when applying the double exponential decay model when differences between the datasets are evident. This suggests that the exponential fitting models proposed here have the potential to correct for the elimination of the fluorescein through the body. However, the results obtained during the implementation of the single and double exponential decay models, did not show enough evidence to allow for accurate prediction of the elimination rates from the fluorescence signals (and therefore, to continue the analysis). This is potentially attributed to the simplicity of the models and the higher level of complexity within the fluorescence signals. Further investigation is needed in order to confidently use the predicted parameters to correct for the elimination phase for comparison between different stages of gut permeability. Therefore, the results from the exponential fitting approaches presented in this section were not taken further in Section 4.3.2 to discriminate between different states of gut permeability. Potential future work in this area is discussed in Chapter 8.



4.3.1.3 Phasor analysis

In contrast to the time-domain methods discussed in previous sections 4.2.3.1 and 4.2.3.2, this last approach analyses the frequency domain of the fluorescence signals. Briefly, the phasor approach focuses on representing the fluorescence signal as a point in a two-dimensional phasor plot. The real part of the phasor component (g) corresponds to the cosine of the phase angle, and it is generally related to changes in the amplitude of the signal, which could be associated with variations in the fluorescence intensity (for example, due to changes in gut permeability). The imaginary component (s), on the other hand, corresponds to the sine of the phase angle and it is usually associated with temporal or phase changes in the signal, and could capture information about the temporal characteristics of the signal. Phasor analysis could, therefore, help to identify differences in gut permeabilities that might be challenging to discern using time-domain methods.

Following this approach, the phasor (real (g) and imaginary (s)) components of the signals were extracted using MATLAB. Previous to this, the fluorescence vs time curves were filtered using a median filter (with window $N=10$). The real component (g) represents the cosine-weighted average fluorescence lifetime, while the imaginary component (s) represents the sine-weighted average lifetime (as represented in Equations 4.6 and 4.7). The phasor components extracted from each signal (shown in Table 4.7) are later use in Section 4.3.2.4 to compare between both fluorescence-only and fluorescence + sugar datasets.

Table 4.7: Phasor components: Real(g) and Imaginary (s) extracted from the fluorescence signals.

ID	Test	Real (g)	Imaginary (s)
P1	F	-0.0581	0.0884
	F+s	-0.0931	0.0752
P2	F	-0.0212	-0.0785
	F+s	-0.0509	0.0887
P3	F	-0.0652	0.192
	F+s	-0.101	-0.0234
P4	F	-0.0297	0.1147
	F+s	-0.024	0.0136
P5	F	-0.081	0.2814
	F+s	-0.03	-0.0874
P6	F	-0.0728	0.0079
	F+s	-0.08	0.1229
P7	F	-0.0215	0.0958
	F+s	0.0028	0.0289
P8	F	-0.0553	-0.028
	F+s	-0.0252	0.042
P9	F	-0.0697	0.0758
	F+s	-0.0478	0.0061
P10	F	-0.0829	0.1288
	F+s	-0.0532	0.0307
P11	F	-0.0463	0.1905
	F+s	-0.0491	0.073
Mean	F	-0.0549	0.0972
	F + s	-0.0501	0.0336
STD	F	0.0226	0.1039
	F + s	0.0314	0.0578

4.3.2 Signal analysis: comparison between different gut permeability stages

4.3.2.1 Fluorescence intensity vs time curves

First step of the comparison between both permeability stages curves involved the evaluation of the fluorescence signals collected from each individual participant on each experimental condition (condition 1: fluorescein only (F) and condition 2: fluorescein + sugar (F + s)).

In both experimental conditions, all 11 participants were asked to fast for a minimum of 5 hours prior to the experiments. During the first phase (condition 1), participants were asked to drink a fluorescein solution containing only 500 mg fluorescein dissolved in 100 ml water, and fluorescence data was collected for 180 minutes using the spectroscopy system. At least 1 week after the first phase, participants came back and were asked to drink another solution containing this time 500 mg fluorescein + 60g of sugar dissolved in 100 ml of water and fluorescence vs time curves were recorded again for 180 minutes (condition 2). Fluorescence intensity vs. time curves from both experiments were plotted to compare the two datasets (Fig. 4.13). It is important to mention that skin tone would of course be expected to affect the collected data, with darker skin tones leading to lower absolute signal levels. However, good signal-to-noise ratios were observed in all measurements indicating that the fluorescein dose used here was sufficient for non-invasive permeability sensing in all cases. In addition, once fluorescence is detected, the normalisation procedure (see section 4.2.1) acts to correct for variations in skin tone (and other factors that affect absolute signal levels such as laser power and probe orientation) between participants.

As expected, when analysing the data, fluorescence intensity was observed to increase over time in both conditions, reaching a peak value approximately within the range of 40-50 minutes after ingestion of the fluorescein solution, before beginning to decrease back towards the background level. Qualitative analysis of the individual fluorescence vs time curves found differences between datasets, with clear variations on the maximum intensities, as well as the intake and decay rates, on each condition. This differences, clearly visible on individuals such as Participants 3, 4, 5, 7, 9, 10 and 11, suggest a change in permeability potentially induced by the ingestion of sugar on the second experimental day. However, there are exceptions in which these observations do not apply. Participants 2, 6 and 8, for example, show higher and early fluorescence intensity peaks for condition 2 (fluorescein + sugar) than condition 1 (fluorescein only). These results can tentatively be attributed to physiological and/or technical variations between experimental studies (as data was recorded with a gap of at least one week). During this period between studies, physiological variations such as gastric emptying rates and gut permeability rates can be altered due to diet, stress, exercise or medication [227, 228]. In addition, unintentional modification during the experimental set up, such as finger clip location and/or laser power variations can also impact the measurements. However, when mean and standard deviation curves were extracted (Figure 4.14), experimental condition 1 (fluorescein only) exhibited a higher peak value (of 0.0046) at earlier times

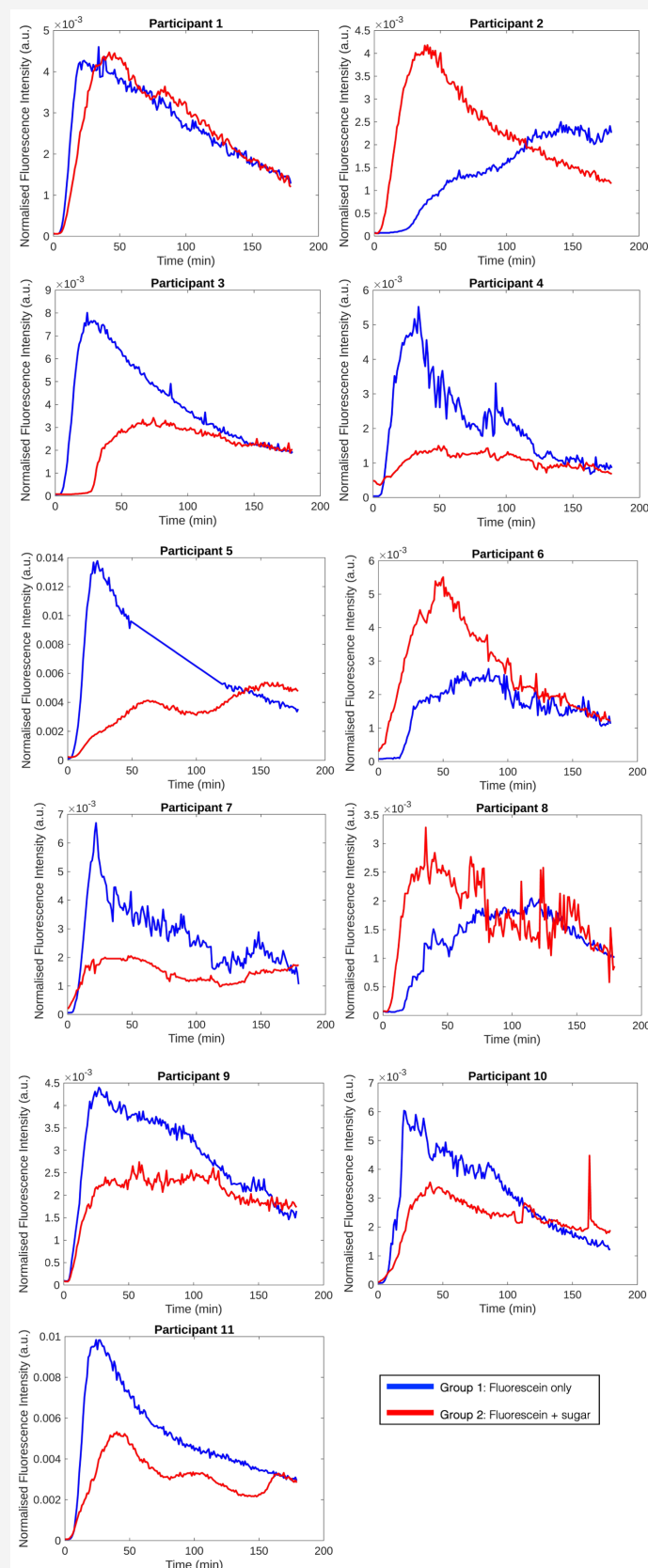


Figure 4.13: Fluorescence vs time curves for all 11 participants under two experimental conditions: fluorescein solution only (blue) and fluorescein solution + sugar (red). Fluorescence signals for each condition were collected on different days using the spectroscopy system displayed in Figure 4.1 for a total period of 180 minutes.

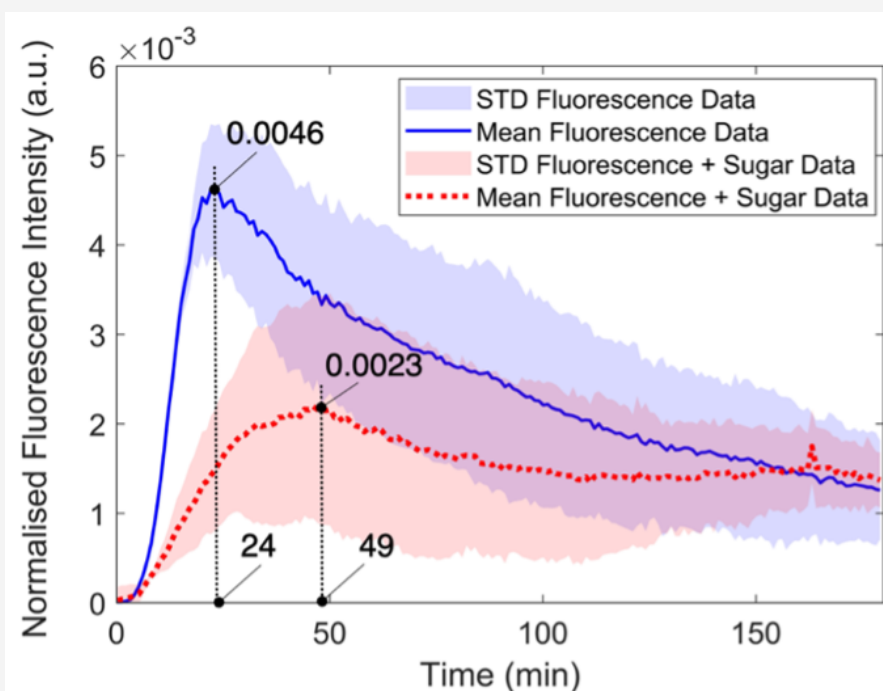


Figure 4.14: Mean fluorescence intensity as a function of time after ingestion of fluorescein for the fluorescein only (solid blue line) and fluorescein + sugar (dotted red line) measurements. The blue and red lines represent mean values calculated across all 11 participants. The shaded blue and red regions represent ± 1 standard deviation from the mean. Peak Time and Peak Intensity values calculated from the mean fluorescence curves are also displayed (not to be confused with the average Peak Time and Peak Intensity values calculated amongst all 11 participants, shown in Tables 4.2, 4.3) and 4.4.

(24 minutes) than the experimental condition 2 (fluorescein + sugar), with a peak value of 0.0023 at 49 minutes.

These findings support the hypothesis that a gut permeability change was induced during the second experimental study, in which 60 g of sugar was ingested together with 500 mg of fluorescein. Nevertheless, differences observed from the individual curves suggest that the ingestion of sugar (during the second study) could have also impacted the gastric emptying rate, slowing it down. This meant not only that the peak signal level occurred later (due to slower emptying of fluorescein from the stomach into the gut) but also that the absolute value at the peak was reduced due to elimination of fluorescein from the blood stream. Fluorescein can be expected to be eliminated from the body at an approximately constant rate across the two measurement days. Thus, it follows that the later the peak in the signal, the greater the degree of elimination that will have taken place by that point, thereby reducing the absolute value of the peak signal. This suggests that parameter correction, such as the Peak AUC/Slope or Peak AUC * Peak Time, could correct for changes caused by alteration in gastric emptying rate and potentially improve the detection of variations in permeability.

4.3.2.2 Classification of different gut conditions - Automatic Peak Selection

Investigation of different manual and computational peak selection approaches (described in Section 4.2.3.1) allowed for the extraction of different parameters - Peak Time, Peak Intensity and Peak AUC - from the fluorescence vs time curves to evaluate the potential of the fluorescence spectroscopy system to detect changes in gut permeability. In addition, we also explore the ability to correct for gastric emptying rate and elimination of fluorescein from the body, by investigating the potential of corrected Peak AUC value (Peak AUC/slope and Peak AUC* Peak time) for a better identification of changes in gut permeability. Figure 4.15 shows the scatter-box plots of the parameters extracted for each participant on each experimental condition with all three different methods.

Using the initial approach (*Method 1*) we found that following sugar ingestion, Peak Time and Peak AUC values were generally higher compared to the fluorescein-only measurements (7 out of 11 for Peak Time and 9 out of 11 for Peak AUC). These differences were statistically significant for both Peak Time and Peak AUC parameters, with p -values = 0.043 and 0.011, respectively.

Conversely, Peak Intensity did not show significant difference (p -value = 0.0811), which could be attributed to the fluorescence intensity variations caused by gastric emptying rates. When analysing the corrected Peak AUC parameters (Peak AUC/Slope and Peak AUC*Peak Time), significant differences were again found between the datasets (p -values = 0.0132 and 0.0031, respectively). This was attributed to the additional degree of correction that the Peak AUC/Slope and the Peak AUC*Peak Time parameters provided for the impact of gastric emptying rates and, potentially, for the elimination of fluorescein from the body.

Table 4.8: Statistical analysis comparing the datasets using the parameters extracted through each method. The p -values obtained from the paired Student's T-test are shown. Statistically significant differences between fluorescence-only and fluorescence + sugar datasets are found in most of the cases, with the exception of parameter Peak Intensity and Peak AUC (for Method 3).

	Peak Time	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
Method 1	0.0403	0.0811	0.0110	0.0132	0.0031
Method 2	0.0403	0.0985	0.0061	0.0161	0.0031
Method 3	0.0439	0.1079	0.3715	0.0159	0.0028

The second method of analysis for peak selection (see Fig. 4.15 and Table 4.8) demonstrated similar results to *Method 1* (manual method), including significant differences between the measurements for Peak AUC/Slope and Peak AUC*Peak Time (p -value = 0.016 and 0.0031, respectively, see Table 4.8).

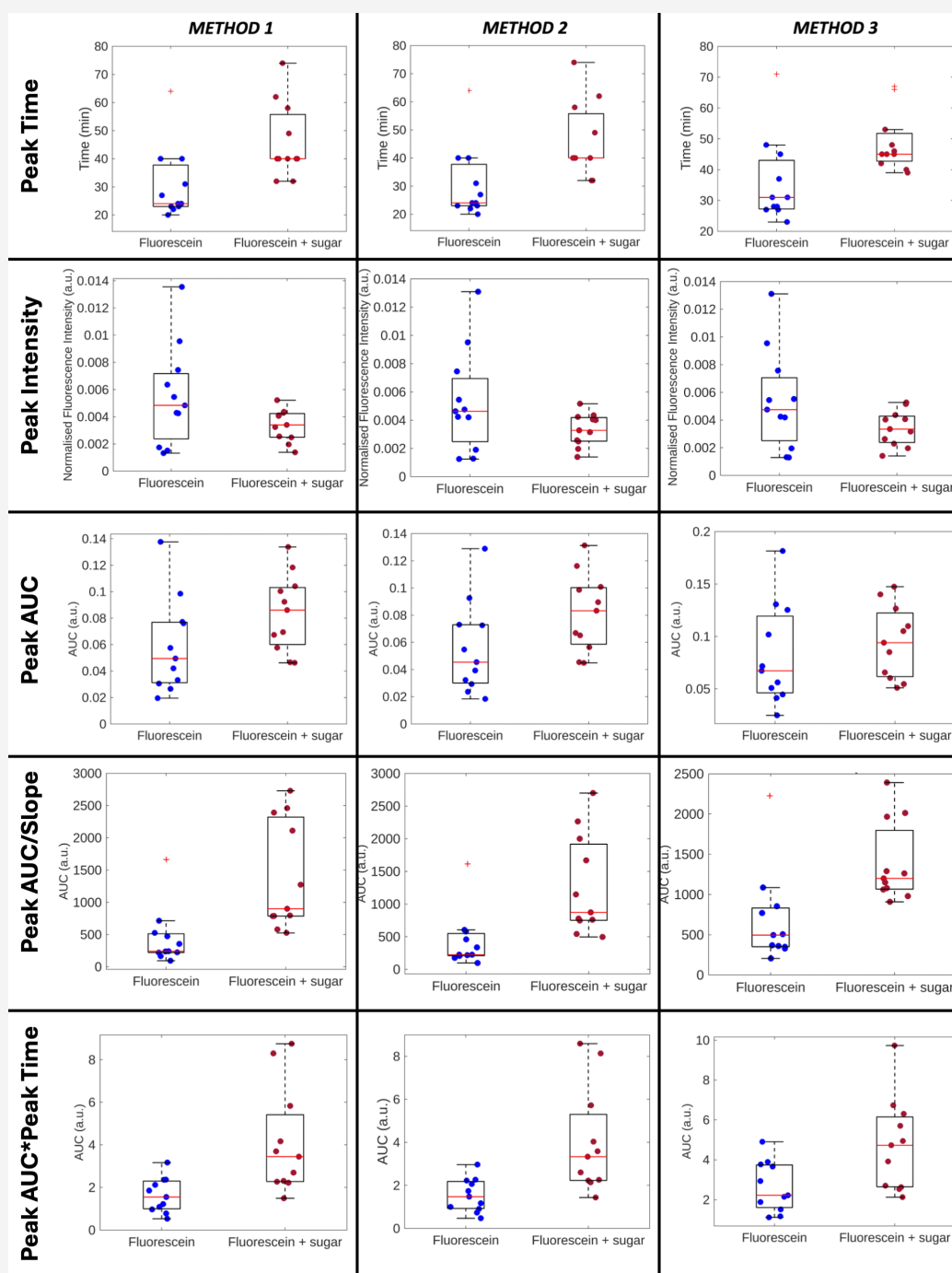


Figure 4.15: Comparison of the parameters extracted from the fluorescence vs time curves. (left to right) Scatter-Box plots showing the parameter values calculated using Method 1, Method 2 and Method 3. (top to bottom) Parameters extracted from the fluorescence curves – Peak Time, Peak Intensity, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time. Box plots also show median values (red lines), 25th and 75th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for the fluorescence only study (blue) and fluorescein + sugar study (red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25th percentile or above the 75th percentile by more than 1.5 times the interquartile range. All calculations (e.g. of means, medians, etc.) and all statistical tests were performed based on the data points from all 11 participants (i.e. including outliers) in all cases.

Interestingly, when analysing *Method 3*, we found that Peak AUC alone did not show significant differences between the fluorescein-only and fluorescein + sugar measurements ($p\text{-value} = 0.371$). However, corrected Peak AUC values (Peak AUC/Slope and Peak AUC*Peak Time) were significantly higher with ingestion of sugar than without ($p\text{-values} = 0.016$ and 0.0028 , respectively).

Furthermore, we found that these Peak AUC/Slope and Peak AUC*Peak Time values were higher following ingestion of sugar in most of the participants for the three methods (9 out of 11 and 10 out of 11 participants for *Method 1*, 8 out of 11 and 10 out of 11 participants for *Method 2* and 10 out of 11 and 9 out of 11 participants for *Method 3*, respectively). Together with the results above, this demonstrates the potential of the parameters extracted, especially the corrected parameters Peak AUC/Slope and Peak AUC*Peak Time, to provide effective/significant discrimination between states of normal and (transiently) increased gut permeability (using all three methods of peak selection analysis). Crucially, this is true both when analysing the data for peak selection in a fully manual manner (*Method 1 and 2*) and when doing so using a completely automated technique (*Method 3*).

This demonstrate that the results for *Method 1* and *Method 2* are not caused by observer/manual bias in the peak selection process. However, it is worth mentioning that the use of unfiltered or noisy data (such as in *Method 1*) can lead to incorrect peak selection and therefore, an unrealistic discrimination. The implementation of a filtering step (such as in *Method 2* and *3*) exhibit similar results to the manual approach (*Method 1*) but reducing the risk of incorrect peak selection, hence, providing an improved analysis of the signals.

Moreover, as data can be analysed immediately following collection, the results presented from this first approach (i.e., parameters extracted from peak selection analysis) highlights the potential for future use in rapid and non-invasive readouts of intestinal permeability. There are of course limitations associated with this peak selection analysis and the approaches generated from it (*Method 1, 2* and *3*). In a small minority of participants, for example, some of the calculated gut permeability parameters (i.e. Peak Intensity, Peak AUC, Peak AUC/Slope and/or Peak AUC*Peak Time) were found to stay the same or decrease following ingestion of sugar (rather than increasing as expected). This was again tentatively attributed to natural variations in physiology (e.g. variations in intestinal permeability, gastric emptying rate and/or rate of elimination/clearance) in the relevant participants from one study day to the next. Nevertheless, the approaches presented here could benefit from advanced techniques that focus on other aspects of the fluorescence vs time curves to improve discrimination between gut permeability stages. Following sections address this consideration, focusing on investigating the potential of more advance analysis to identify features that will improve the discrimination between different gut permeability stages.

4.3.2.3 Classification of different gut conditions - Exponential decay fitting

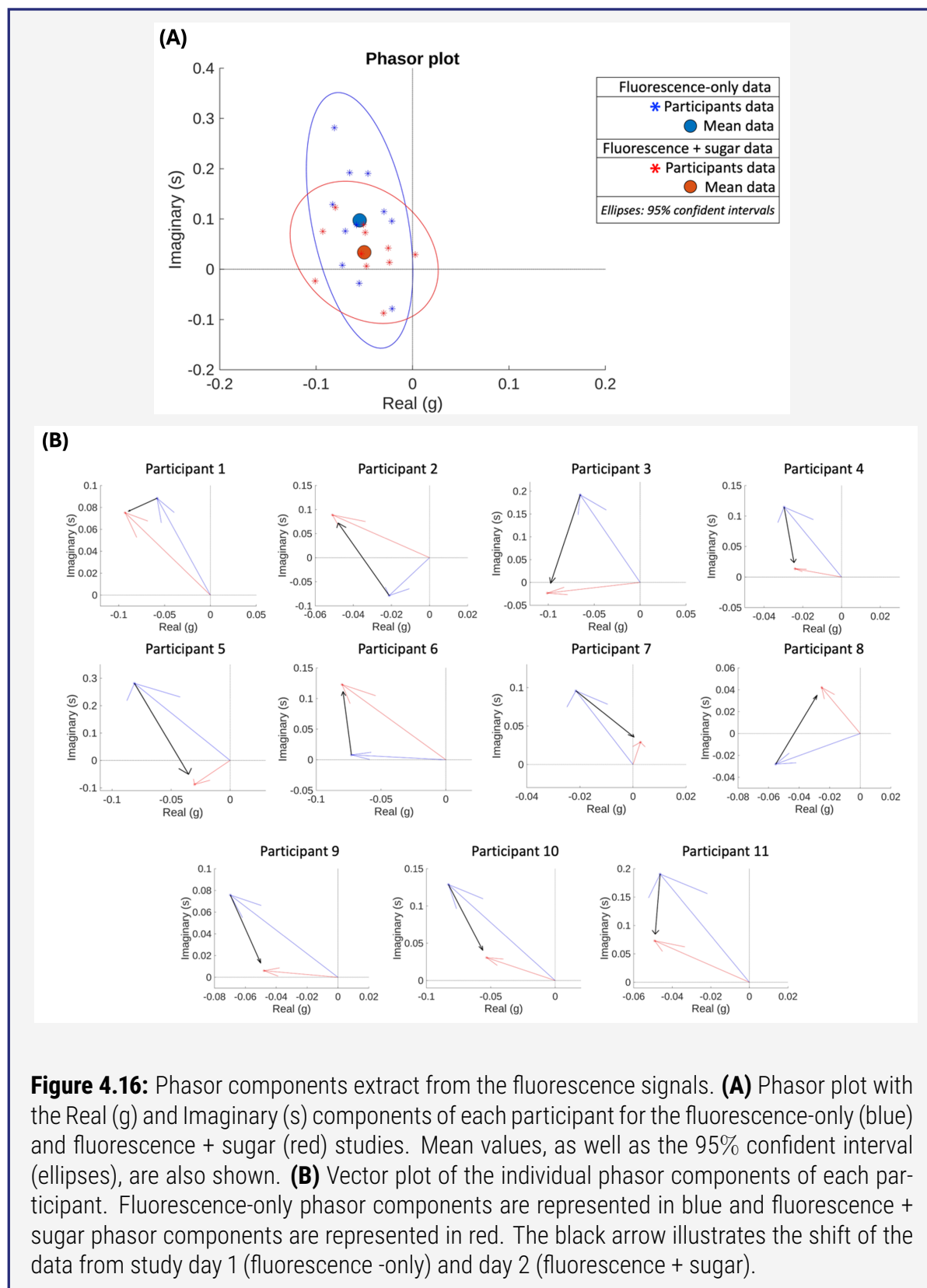
As observed in Section 4.2.3, the fluorescence vs time curves collected using the spectrometer system exhibit three characteristic phases: absorption, maximum intensity and clearance. Parameters extracted in the previous section show good discrimination between the datasets. However, given the potential impact of the gastric emptying rates on signal dynamics, further analysis focused on investigating the influence of other factors, such as the fluorescein elimination (clearance phase), on dataset discrimination. To accurately describe and correct for the elimination of the fluorescein from the bloodstream (clearance phase), an exponential decay model is fitted to the fluorescence vs time curves to obtain a decay rate. The calculated decay rates are then used to correct the fluorescence signals. We saw in Section 4.3.2.3 that the accuracy of the exponential decay fitting was very dependent of the started point selected for the model. In addition, some of the fluorescence signals do not exhibit a clear exponential decay region for the models to work with. These limitations generated unreliable prediction of the decay rates, resulting in unrealistic corrections of the fluorescence signals. Therefore, the results from this approach were not considered for comparison between datasets and further investigation is needed in order to confidently use the predicted parameters to correct for the elimination of fluorescein in the body. Future goals involve refining the exponential decay fitting algorithms by incorporating constrains, including expected parameter values for decay rates, to improve the accuracy of the prediction and thus, the correction for fluorescein elimination in the body. Furthermore, exploring addition features, such as the uptake of the fluorescence intensity, could also improve the characterisation of the fluorescence curves and facilitate the discrimination between gut permeability stages.

4.3.2.4 Classification of different gut conditions - Phasor analysis

Following with the implementation of advance analysis approaches, in Section 4.3.1.3 we extracted the phasor (real(g) and imaginary (s)) components from the fluorescence signals to investigate the capability of the phasor analysis to discriminate between different gut permeability stages. Figure 4.16 shows the phasor components of each individual participants and study, as well as the mean values for each group. Interestingly, after sugar intake, the mean phasor component exhibits a downward shift in position with respect to the fluorescence-only data, with a Euclidean distance of 0.0637. This downward deviation of the fluorescence + sugar signals is also noticeable in most of the individual signals (Figure 4.16), suggesting, at first, a change in the gut permeability caused by the intake of sugar.

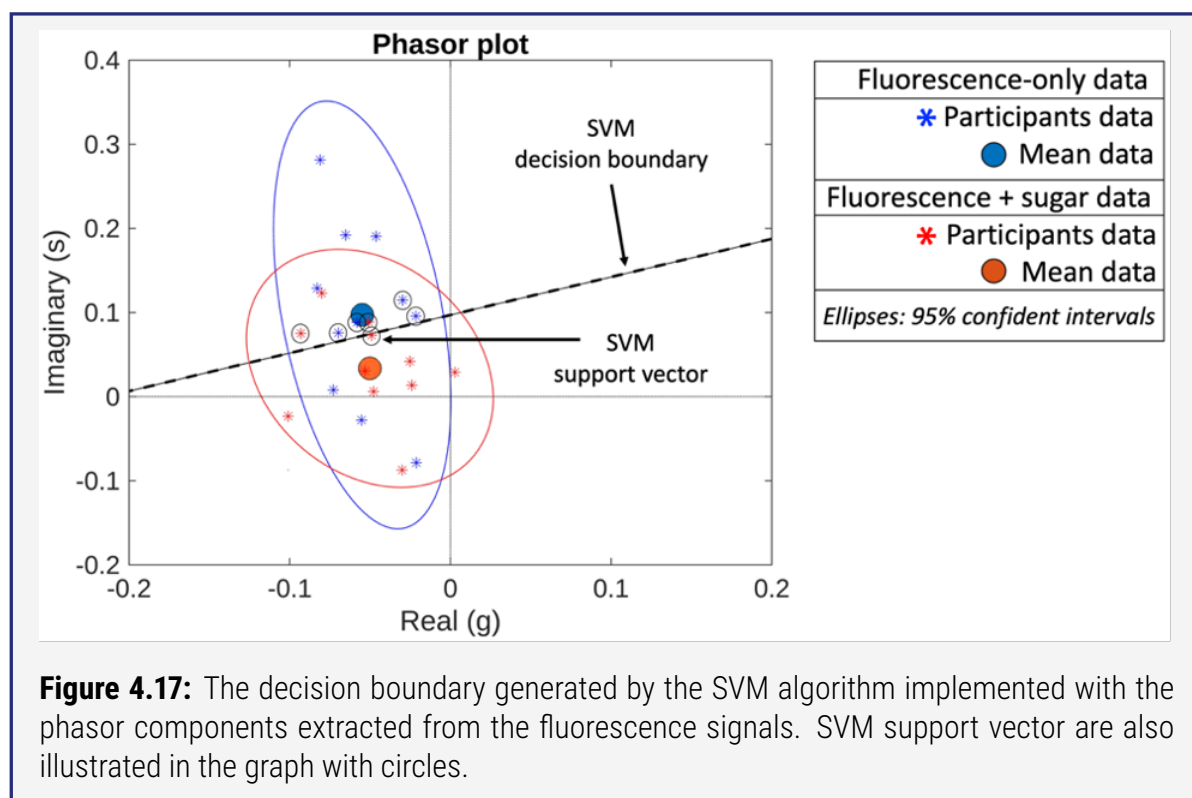
Interestingly, although this downward shift observed between the individuals was found to be non-significant (p -value: 0.0659), participants which exhibited an upward shift (Participants 2, 6 and 8) also showed higher fluorescence + sugar intensity signals (Fig. 4.13). In addition, this phenomenon also correlates with the findings in Section 4.3.1.1 and 4.3.2.2, in which these participants exhibited higher fluorescence + sugar Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time values (see

Tables 4.2, 4.3 and 4.4). As before, these observations were rather mainly attributed to natural variations in physiology (e.g. variations in intestinal permeability, gastric emptying rate and/or rate of elimination/clearance) from one study day to the next.



Nevertheless, to further investigate the potential of the phasor analysis to discriminate between gut permeability stages, we integrated the phasor components extracted from the signals into a machine learning algorithm. In this case, we built a preliminary/simple supervector machine (SVM) classifier to distinguish between both datasets and we use the phasor components extracted from the fluorescence signals as inputs. The SVM algorithm is widely used to capture complex relationships within different datasets [225]. Its objective is to find the optimal hyperplane that maximises the separation between the data points within these groups. To do so, data, containing the phasor components is randomised and divided into two groups: training and validation (containing 70% of the data points) and testing (containing 30%). The training and validation data is then implemented into a simplify SVM is using the MATLAB function *fitcsvm*.

To optimise the parameters within the SVM algorithm and to assess the performance of the SVM algorithm, we use the “leave-one-out” cross validation (LOOCV). In this technique, a single data point from within the training and validation group is held out as the validation set, and the model is trained in the remaining data. This process is repeated for each data point in the training and validating group. This ensures that every data point serves as both part of the training set and the validation set, allowing for a robust assessment of model performance with limited data. After the validation, the data points within the testing groups are used to assess the final performance of the SVM model. Figure 4.17 exhibit the decision boundary generated by the SVM after the testing and the support vectors that influenced the position and orientation of the decision boundary.



The confusion matrix (Table 4.9) obtained after the implementation of the algorithm describes the performance SVM model. As observed in table 4.9, only one participant was misclassified (out of the 6 total participants used to evaluate the overall performance of the classifier). Given the confusion matrix, the resulting SVM model provided an overall accuracy of 83.333%, with an out-of-sample misclassification (or validation error) of 21.439% (obtained from the LOOCV). Given the limited data available, the sensitivity of the model, 66.666%, suggests a good discrimination between fluorescence-only and fluorescence + sugar signals. This is also supported by the F1 Scores, 94.123%.

Table 4.9: Confusion matrix extracted from the SVM algorithm. These values are used to calculate the performance metrics of the algorithm: accuracy, sensibility and F1 score.

True Class	Fluorescence + sugar	Fluorescence-only
	3	2
Predicted Class	Fluorescence + sugar	Fluorescence-only
	3	0

Overall, the results presented in this section demonstrates the potential of the phasor components together with the SVM classifier to discriminate between different stages of gut permeability. However, it is important to highlight that the results provided by the SVM model do not represent the overall picture of the fluorescein permeability profiles. With limited data available, the SVM model is more susceptible to overlook the complexity of the data, which can let to overfitting or biased representations. Therefore, the SVM model presented here served as a foundational step to promote the development of more complex strategies in the future to capture the full complexity inherent in the fluorescence signals and to showcase the potential of the phasor components to discriminate between different gut permeability stages.

4.4 Conclusion

This chapter has reported a series of analysis techniques for assessment of intestinal permeability using non-invasive (transcutaneous) fluorescence spectroscopy. Our first approach (Peak Selection) demonstrated that three methods of analysis (ranging from manual to fully automated) produce similar results for the parameters investigated. In addition, the parameters extracted from the fluorescence signals provided significant difference between the fluorescence-only and fluorescence + sugar datasets, allowing for an effective assessment and comparison of different stages of gut permeability.

Further advanced techniques investigated the potential of new features such as the clearances rate to correct for the elimination of the fluorescein from the body and the impact this has on the fluorescence signals. However, the exponential decay fitting approach investigated in this case, although promising, did not provide feasible results for the correction of the elimination rates. Given the high dependency on user input, the decision was made to refrain/abstain from implementing this approach in the subsequent chapter, recognising the need to further work.

The last approach analysed the complex frequency domain of the fluorescence signals (phasor analysis). The results demonstrated the potential of the phasor components to effectively discriminate between different stages of gut permeability, allowing for a rapid and automated (no external/human input) analysis of the signals. Our results demonstrate the ability of the parameters (extracted through the Peak Selection and Phasor analysis approaches) to differentiate between two states of gut permeability. In addition, the significant differences found between the datasets when implementing these approaches reveals the potential of transcutaneous fluorescence spectroscopy for non-invasive assessment of intestinal permeability.

As permeability is known to be altered in a wide range of GI and other conditions (e.g. in Crohn's disease, coeliac disease and malnutrition [143]), this suggests that transcutaneous fluorescence spectroscopy may have clinical potential, for example in both diagnostic and therapy/intervention monitoring applications. Crucially, the fluorescence spectroscopy method reported here is non-invasive, does not require collection of biological samples such as blood or urine, and involves analysis procedures, such as Peak Selection and Phasor analysis, that can be performed in a fully automated manner. Furthermore, the fluorescence spectroscopy technique uses fluorescein as a contrast agent, which is low-cost and already approved for other clinical procedures [185, 190]. Together, this suggests significant advantages over existing permeability tests (e.g. L:M tests, Cr-EDTA assays, etc.) in terms of both invasiveness and time to report results which implies the potential for rapid clinical translation. In turn, this technique, together with the computational approaches presented, would offer opportunities to improve patient outcomes in some of the above conditions, for example by providing earlier diagnosis and improved monitoring of therapy responses.

There are of course limitations associated with this study. The sample size is relatively small and

there is also a risk of heterogeneity in gut permeability among the study participants (both in their gut permeability across the two study days and in how they respond to the hyperosmolar solution). The gut is an extremely complex organ, which exhibits functional changes in response to subtle and wide-ranging factors such as diet and exercise. As such, it is not unexpected that the data exhibited variations across participants. Nonetheless, this highlights the need to further validate this technology in larger cohorts/populations.

Furthermore, only Asian and Caucasian volunteers participated in this study (see Table 4.1), meaning that the range of skin tones investigated was limited and is expected to impact the collected signals. However, while further investigation of the effect of skin tone would be beneficial, the results presented here still demonstrate the potential of transcutaneous fluorescence spectroscopy for non-invasive permeability sensing.

To address the above limitations, the following chapters present a compact portable version of the benchtop system (used in this chapter) to allow more widespread deployment in larger clinical trials. In addition, this technique is also validated in larger populations (with a wider range of skin tones and with different doses of fluorescein) and in patient cohorts where increased intestinal permeability is expected (e.g. potential undernutrition participants). Together with the results reported here, this will further validate transcutaneous fluorescence spectroscopy as a clinical tool for non-invasive monitoring of intestinal permeability.

Chapter 5

Fibre-optic fluorescence spectroscopy sensor - development and validation

As discussed in Chapter 4, permeation of the contrast agent, fluorescein, from the gut to the blood-stream is detectable through the skin using fluorescence spectroscopy. In addition, fluorescence datasets, together with different data processing approaches, have shown to have the potential to be clinically useful – for example in the monitoring and diagnosis of abnormal gut permeability. Previous studies also helped to provide evidence that fibre-optic fluorescence spectroscopy systems, firstly presented in [90], could prove useful in the investigation of other intestinal functions, such as gastric emptying, as well as other GI disorders, such as IBD or Chron’s disease [90, 145, 209, 229, 230]. These spectroscopy systems can significantly improve the experience of patients while also providing the opportunity to clinicians with valuable information. However, when considering the non-invasive monitoring and diagnosis of gut disorders using optical spectroscopy, it is important to consider the accessibility and deployability of the technology. This chapter describes the development of a compact fluorescence sensor designed to improve the functionality of the system present in previous chapters and referred to as *Benchtop system*. This compact sensor takes into account the limitations of this system, focusing on deployability and cost-effectiveness. The presented sensor integrates a compact in-house electronic design that allows for collection of fluorescence data non-invasively using a single excitation source and a fibre optic probe. The system is described in detail below, including the development, testing and validation strategies implemented. The information provided in this chapter has been published in the SPIE Journal of Biomedical optics (*open access*) [230].

5.1 Introduction

As previously explained, disruption of the intestinal barrier manifests in numerous gastrointestinal (GI) conditions and can lead to an increase in gut permeability, potentially allowing GI pathogens to enter the bloodstream and trigger inflammatory responses that exacerbate further the condition [27, 29, 90, 143, 145, 231].

Despite the biological and clinical importance of intestinal permeability, we have seen that current clinical tests suffer from a series of limitations, including invasiveness, lack of standardisation and high cost. The clinical uptake of these tests is therefore constrained to certain population [143, 144] and regions, which demonstrates the need for non-invasive and reliable diagnostic tools for improved assessment of intestinal permeability [36, 89, 142].

In Chapter 3 and 4, we have seen that transcutaneous ('through-the-skin') fluorescence spectroscopy of orally ingested fluorescent contrast agents has the potential for non-invasive monitoring of gut permeability and other GI functions [90, 145–148, 209]. Fluorescein, a clinically approved fluorescence contrast agent [190], possesses the capability for transcutaneous assessment of gut function as it has a molecular weight that is comparable to lactulose (which is used in the L:M and L:R tests). The use of transcutaneous spectroscopy has the potential to produce clinical results within hours rather than days (as data can be analysed immediately without requiring laboratory analysis) and does not require collection of urine, blood or stool samples (unlike other methods such as L:M and L:R) [90, 145–148, 209]. However, to date, transcutaneous fluorescence spectroscopy to measure gut permeability has only been deployed clinically using laser-based spectrometers and microscopes (hereafter referred to as benchtop systems), which are both large and expensive [90, 145, 146, 209]. For example, a preliminary estimate of the average cost of the benchtop system was calculated at £ 10,000. The use of these benchtop systems can therefore introduce additional challenges in certain patient groups (e.g. children/infants) and in certain environments (such as low-income countries), thereby precluding widespread deployment.

To address these issues, this chapter presents a compact fluorescence sensor for non-invasive assessment of gut permeability via transcutaneous spectroscopy. The performance of the compact optical sensor is compared against a clinically deployed benchtop system that was previously presented in the literature [90, 145, 209] and in Chapter 4. In addition, this chapter reports preliminary data from a clinical study on healthy participants investigating the impact of skin tone and contrast agent dose on transcutaneous fluorescence signals, thereby demonstrating the potential of the compact sensor for non-invasive monitoring of gut function.

5.2 Methods

5.2.1 Benchtop System – control system

To analyse the performance of the developed portable fluorescence sensor and to investigate the potential of the device to monitor gut function, an existing benchtop spectrometer (presented in Chapter 4) was used as a control system. The benchtop system – which was originally reported in Ref. [90]– comprises two laser sources (Stradus 488-25 and Stradus 785-80, Vortran Laser Technology, USA) for excitation of fluorescence and a compact spectrometer (FLAME-S-VIS-NIR-ES, Ocean Insight) for detection. Bandpass and neutral density (ND) filters are used to clean up the laser emission profiles and to limit the optical excitation power to safe levels. Long pass filters (housed within a motorized filter wheel) are used to reject backscattered excitation light. Finally, a bifurcated optical fibre probe (QR200-7-VIS-NIR, Ocean Insight) is used for light delivery and collection to allow interrogation of fluorescence signals at the skin. 488 nm excitation was used for all experiments presented in this article, as this provided efficient excitation of fluorescein fluorescence (the fluorescent contrast agent used for all experiments) and was comparable to the excitation wavelength of the compact fluorescence sensor (465 nm).

The benchtop system collects a fluorescence spectrum at every time point and converts the spectrum into a scalar value to represent fluorescence intensity. This is carried out by summing over the wavelength range of 500-580 nm (containing the spectral peak of fluorescence signal) and then normalising the integrated fluorescence value according to both integration time and laser power (see further details in Ref. 10). Previous studies using this benchtop system have shown the potential of transcutaneous fluorescence spectroscopy for non-invasive assessment of gut permeability and gastric emptying rate [90, 145, 209]. Hence, the benchtop spectrometer represented a suitable comparator system for validation of our novel, compact fluorescence sensor.

5.2.2 Instrument development

This section provides a description of the development of the compact fluorescence spectroscopy sensor, including the optical and electronic configuration, the control software, the safety considerations, and the methods used for data analysis. The individual sub-sections present information regarding the following: 5.2.2.1– optical set-up; 5.2.2.2 – custom optical fibre-probe used for light delivery and collection; 5.2.2.3 – electronic configuration; 5.2.2.4 - data normalization; 5.2.2.5 – home-written control software; 5.2.2.6 – safety considerations.

5.2.2.1 Compact fibre-optic fluorescence sensor – optical set-up

As discussed above, transcutaneous fluorescence spectroscopy entails the oral ingestion of a fluorescent contrast agent (the clinically approved dye fluorescein was used in all experiments presented here) and the non-invasive measurement (using a sensor placed in contact with the skin)

of the permeation of that contrast agent from the gut into the blood stream. The resulting data can then be analysed to facilitate measurements of gut permeability and other clinically relevant GI functions.

To allow more widespread deployment of transcutaneous spectroscopy to assess gut permeability, a compact fluorescence spectroscopy sensor was developed to evaluate fluorescence signals at the fingertip. While compact and even wearable transcutaneous fluorescence sensors exist (Ref. [149]), a fibre-based system has been developed here to facilitate the closest possible spacing between light source and detector. This provides the highest possible sensitivity, which is crucial for assessment of gut function where the concentration of fluorescent dye in the blood stream is typically low compared to applications in other organs (e.g., in the kidney or liver where dyes are injected into the blood stream).

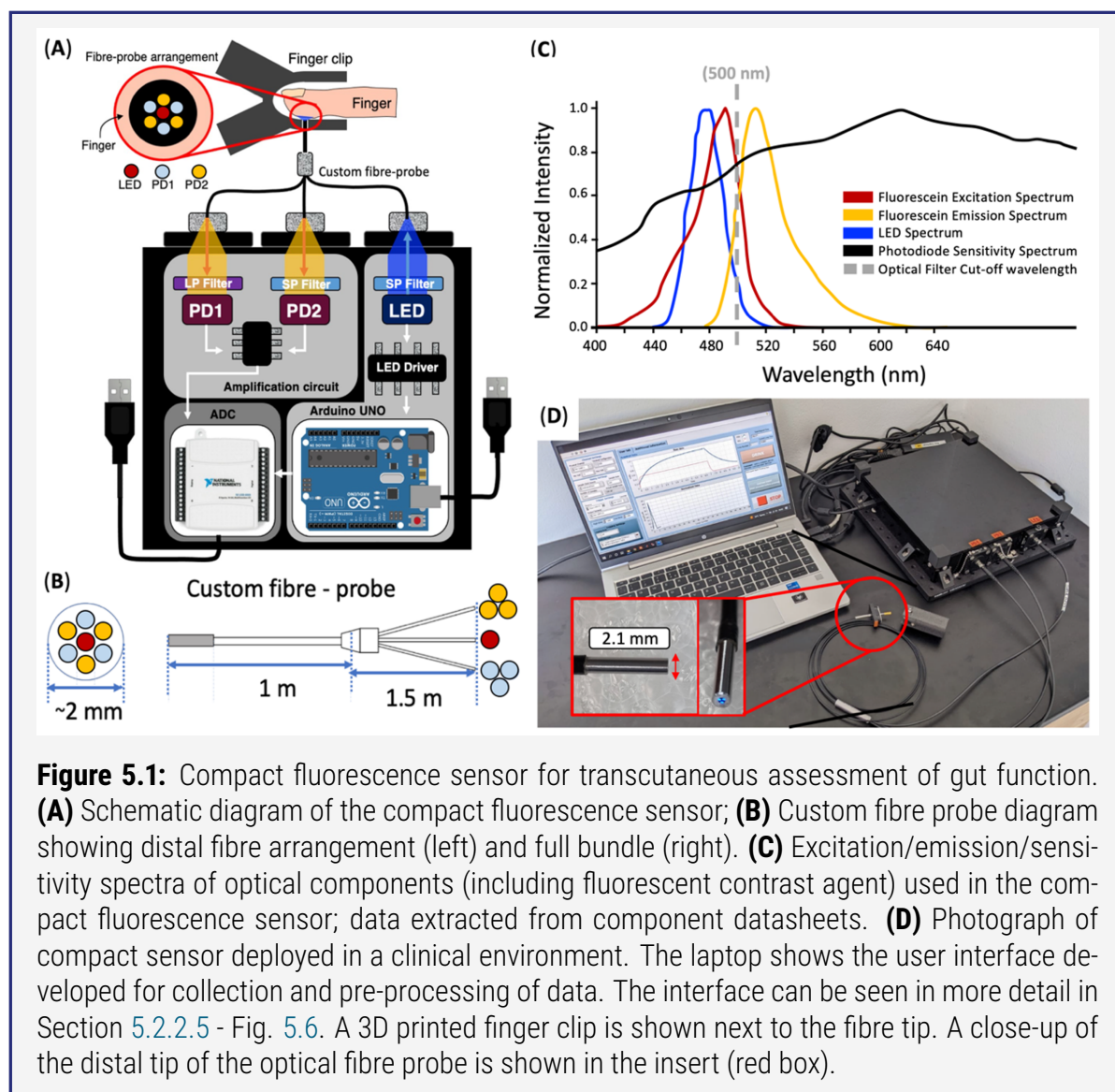
A schematic diagram of the optical setup for our compact sensor is shown in Fig. 5.1A. The optical part of the sensor comprises a 465 nm light emitting diode (LED; LST1-01H06-BLU1-01, New Energy) for excitation and two Photodiodes (2 x SFH 2716 A01, ams OSRAM, New Energy; peak sensitivity at 620 nm, 80% relative sensitivity at 520 nm) to detect the fluorescence signal (Photodiode 1) and the backscattered excitation signal (Photodiode 2). From now on, the Photodiodes will be referred to as the Fluorescence Photodiode (Photodiode 1) and the Backscatter Photodiode (Photodiode 2). The signal collected by the Backscatter Photodiode is used to correct for variations due to, for example, changes in probe orientation, excitation power fluctuations, and differences in skin colour and/or skin thickness.

The light source (LED) and the detectors (Photodiodes) are coupled to a custom trifurcated fibre-optic probe (2 m total length; FiberTech Optica Inc.) to allow interrogation of fluorescence signals at the skin (Fig. 5.1B). The custom probe contains 7 multimode silica optical fibres (200 μm core diameter). A detailed description of the arrangement of fibres in the custom probe is explained in Section 5.2.2.2.

Flexible reflective optical filters (Everix Ultra-Thin OD 2 Longpass/Shortpass Filters, cut-off/on wavelength: 500 nm, Edmund Optics) were positioned in front of the LED and the Photodiodes. Short pass filters were used to clean up the LED emission profile and to stop any fluorescence signal from reaching the Backscatter Photodiode. Similarly, long pass filters were used to stop backscattered excitation light from reaching the Fluorescence Photodiode. The spectral properties of the optical components are presented in Fig 5.1C.

Two filters were used in front of each component (i.e. LED and photodiodes) to ensure sufficient light rejection. Fig 5.2 shows the suppression of excitation light achieved using optical configurations with one and two short and long pass filters respectively. Graphs in Fig. 5.2 show spectra observed when LED excitation light was directed through the optical filters onto a spectral detector (FLAME, Ocean Insight). While the use of one short pass and one long pass filter resulted in clear detection of residual signal from the LED, the two-filter configuration provided near-total suppres-

sion of the excitation light (Fig. 5.2). For this reason, a two-filter configuration was used for all subsequent experiments.



Finally, the distal tip of the custom fibre probe is attached to the forefinger of the participant using a 3D-printed finger clip. This finger clip was designed with a spring-loaded mechanism that ensures constant pressure and holds the probe in contact with the skin for the duration of the measurements, thereby minimising motion artifacts, while also providing comfort to the participant. To drive the sensor, a custom-written LabVIEW (National Instruments, USA) interface (described in more detailed in Section 5.2.2.5) controls all components and provides automatic pre-processing of the collected data. The entire device (including the sensor, fibre probe, finger clip and laptop running LabVIEW control software) is shown in Fig 5.1D.

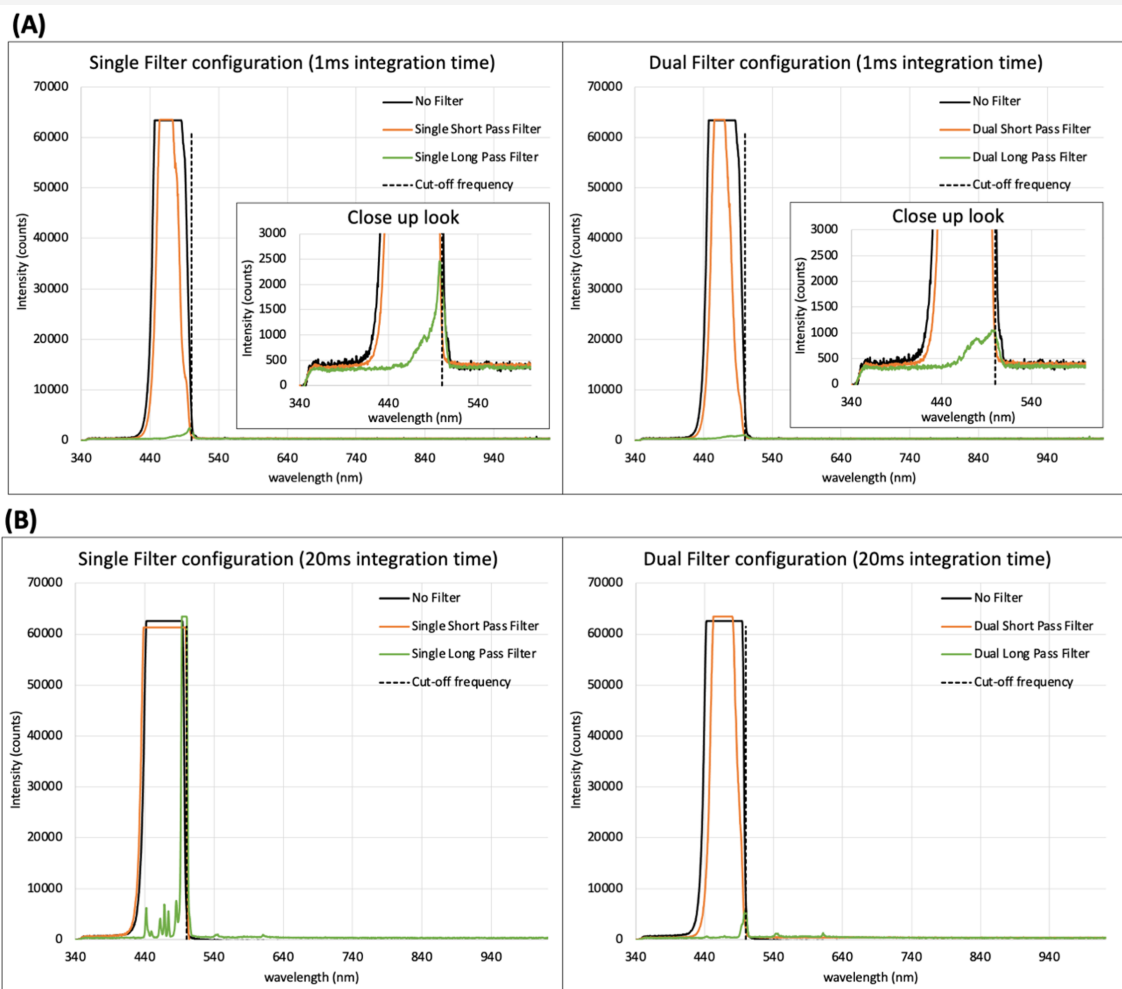


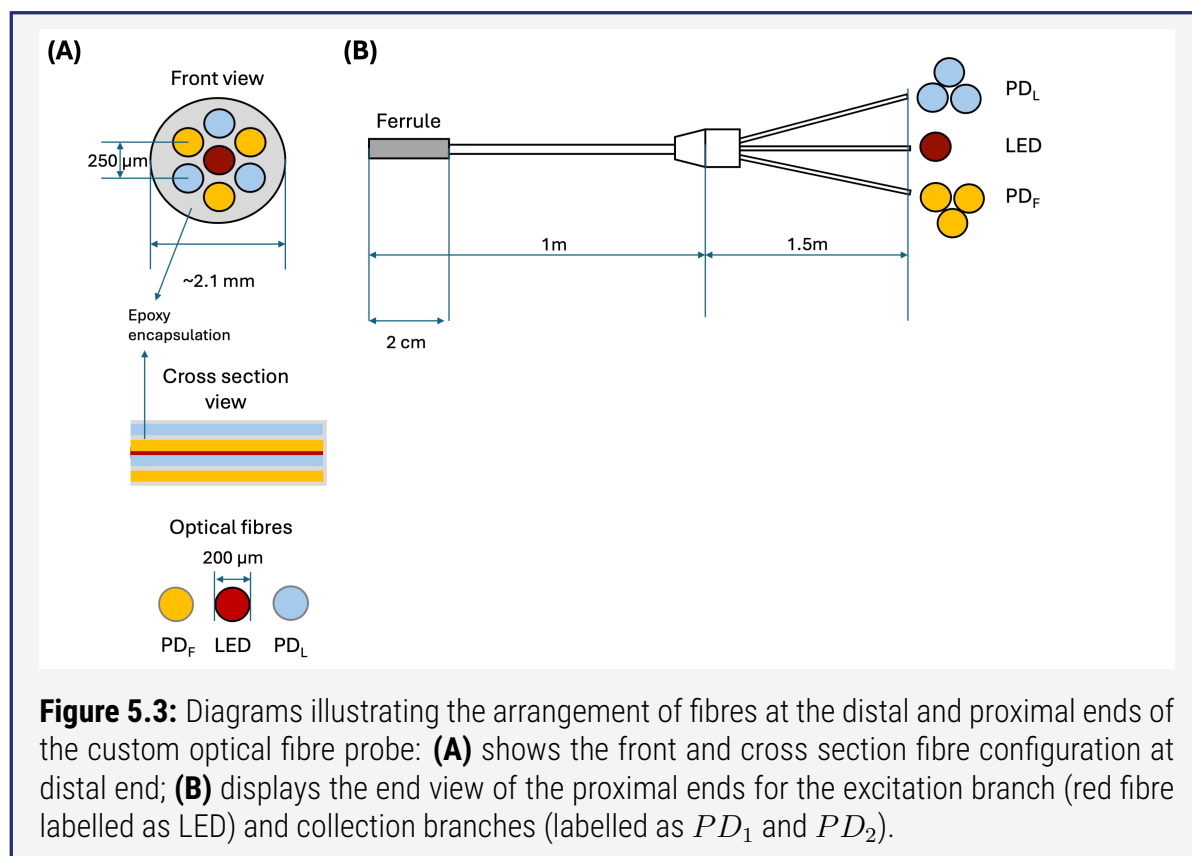
Figure 5.2: Light suppression comparison between single and dual filter configuration. Graphs show spectra collected using a FLAME spectrometer (Ocean Insight) when LED excitation light (465 nm central emission wavelength) was directed through long pass and short pass filters (with cut-off frequency at 500 nm) into an optical fibre probe connected to the spectrometer. **(A)** Spectra acquired for single (left) and dual (right) filter configurations with 1 ms integration time. Inset graphs show close-up views of the spectra. **(B)** Spectra acquired for single (left) and dual (right) filter configurations with 20 ms integration time. For both integration times, the residual LED signal is considerably lower when using the dual filter configuration as compared to the single filter configuration.

5.2.2.2 Compact fibre-optic fluorescence sensor – detailed description of the custom fibre-probe

As previously mentioned, a custom-built fibre-optic probe (manufactured by FiberTech Optica Inc.) comprising multimode optical fibres (all of 0.22 NA and 200 μm diameter) is used for the excitation and collection of fluorescence signals. This custom fibre probe has a single distal branch (integrating 7 optical fibres) and a proximal end divided into three individual branches.

In the common distal end of the custom probe, a single excitation fibre, located in the centre of

the optical fibre bundle, delivers the light for the fluorescence excitation of the contrast agent (fluorescein). This excitation fibre is surrounded by a ring of 6 fibres that collect the fluorescence and backscattering light. These collection fibres are interleaved at the distal tip to ensure that the interrogation volume is the same for both Photodiodes. The centre-centre spacing of fibres at the distal tip is 250 μm , which is the same as that in the Benchtop system [1-3] used for validation (see Section 5.2.1 below). Figures 5.1 and 5.3 illustrated the configuration of the fibres in distal end of the custom fibre-probe. The LED excitation fibre is coloured in red; the fluorescence collection fibres are blue and the backscattered light detection fibres are shown in yellow. The optical fibres are hexagonally packed and secured using epoxy inside a 2 cm stainless-steel ferrule. No protrusion is left on the stainless-steel encapsulation beyond the distal face of the fibre bundle to ensure that all fibres are always in contact with the surface of the skin. This is illustrated in Fig. 5.3A which shows a cross section of the distal end of the custom probe. The total outer diameter of the distal branch is approximately 2.1 mm. Because the fluorescence signals are non-invasively collected from the surface of the skin (typically the fingertip), no waterproof or sterilization requirements were considered during the assembly of the custom probe. However, the distal and proximal parts of the probe are resistant to alcohol, which allows for easy and rapid cleaning between uses.

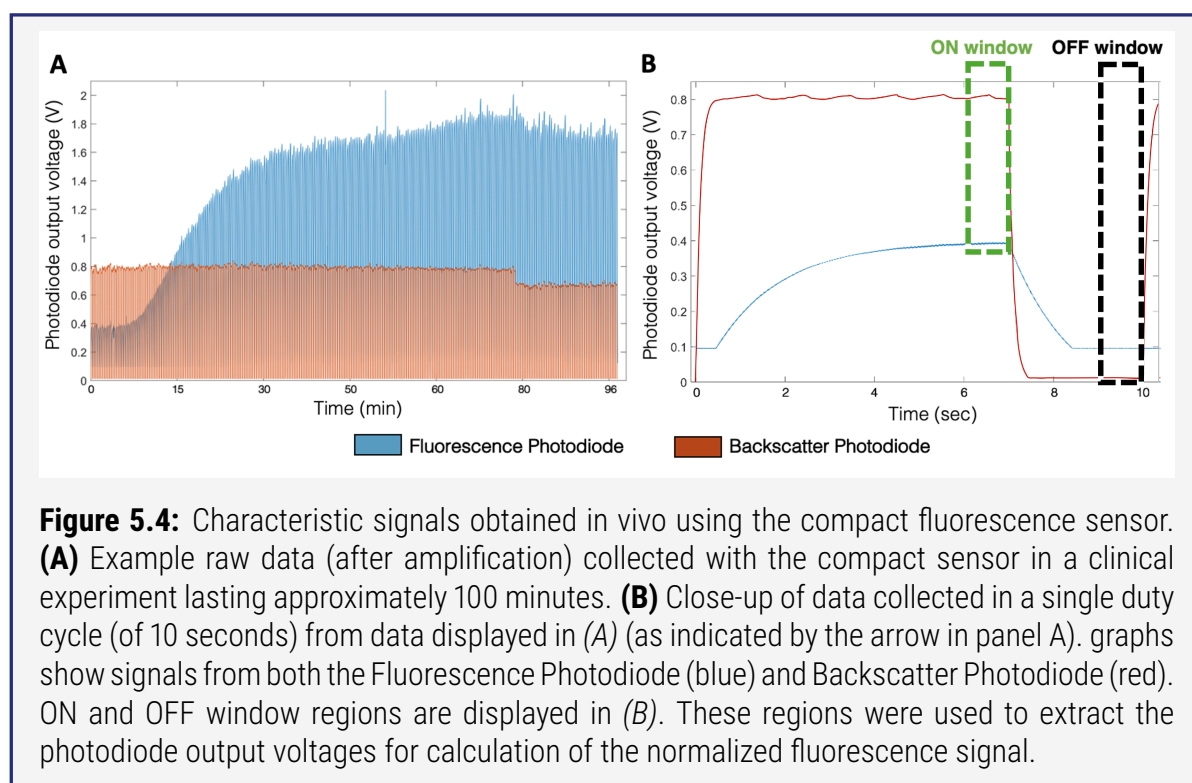


The proximal end has 3 independent channels that are connected to the LED for excitation and the two Photodiodes for detection, respectively (Fig. 5.3B). The single optical fibre routes the light from the LED to the measurement site (typically the fingertip). Of the ring of 6 collection fibres, 3 are connected to the Fluorescence Photodiode and 3 are connected to the Backscatter Photodiode.

As in the distal part of the custom probe, the centre-centre spacing of the fibres is 250 μm . SMA connectors are mounted on the proximal end of each fibre bundle to securely connect the fibres with the electronics. This also ensures the alignment of the fibres with the LED light and detectors, so when the excitation and detection fibres are connected, they are aligned with the centre of the sensitive area of the LED and photodiodes, respectively, as shown in Fig. 5.1A.

5.2.2.3 Compact fibre-optic fluorescence sensor – electronic configuration

The sensor electronics were powered and controlled using an Arduino UNO and a USB data acquisition controller (DAQmx – NI USB-6289, National Instruments), both of which were connected via USB to a laptop computer. The electronic design of the sensor was guided by the input signals detected by the photodiodes. Fig. 5.4 shows an example of a typical raw output signal collected with the sensor in a clinical study participant. As light illuminates the skin, the Photodiodes detect the fluorescent and backscattered signals (Fig. 5.4, blue and red curves respectively). As the concentration of contrast agent in the bloodstream increases, the Fluorescence Photodiode signal increases (Fig. 5.4A, blue).



Without amplification, the photodiode currents generated by the fluorescence emission are very low (on the order of 0.223 μA). This implies the need for a high electronic gain on the Fluorescence Photodiode to produce a measurable output signal. For this reason, power gains of 110.737 dB and 90.414 dB were respectively applied to the Fluorescence and Backscattered Photodiodes using two-stage amplification. These gain values were determined via preliminary in vitro experiments and computational simulations performed using LTSpice.

Initially, a fluorescence vs time curve, derived from a patient, obtained during a previous in vivo study using the fluorescence spectroscopy system (described in Section 5.2.1), served as the basis for extracting the time point corresponding to maximum fluorescence intensity. Subsequently, the time spectrum at this identified point was corrected using the following equation:

$$\text{Fluorescence Intensity} = \frac{\text{Time curve} - \text{mean background signal}}{\text{aq. time (seconds)}} \quad (5.1)$$

The corrected time spectrum was then used to characterize the spectra of various fluorescein solutions, which contained fluorescein at concentrations 0 mg/ml (i.e., water with no fluorescein), 0.00044 mg/ml, 0.00056 mg/ml, 0.00083 mg/ml and 0.0017 mg/ml. Upon comparison, the fluorescein solution that exhibited closest resemblance to the corrected time spectrum (extracted from a patient) was evaluated with a photodiode to quantify the output current, yielding a value of 0.223 μA . An initial circuit design (depicted in Fig. 5.5A) was recreated in LTSpice. The amplification values (i.e., R_f , R_2 and R_3) used on the LTSpice computational simulations were used as a foundational point for the electronic design.

The final circuit design featured a two-stage amplification as the required total power gain values of 100.737 dB (for the Fluorescence Photodiodes) and 90.414 dB (for the Backscattered Photodiodes) exceeded those achievable with a single amplifier. These gain values were kept constant for all experiments presented herein.

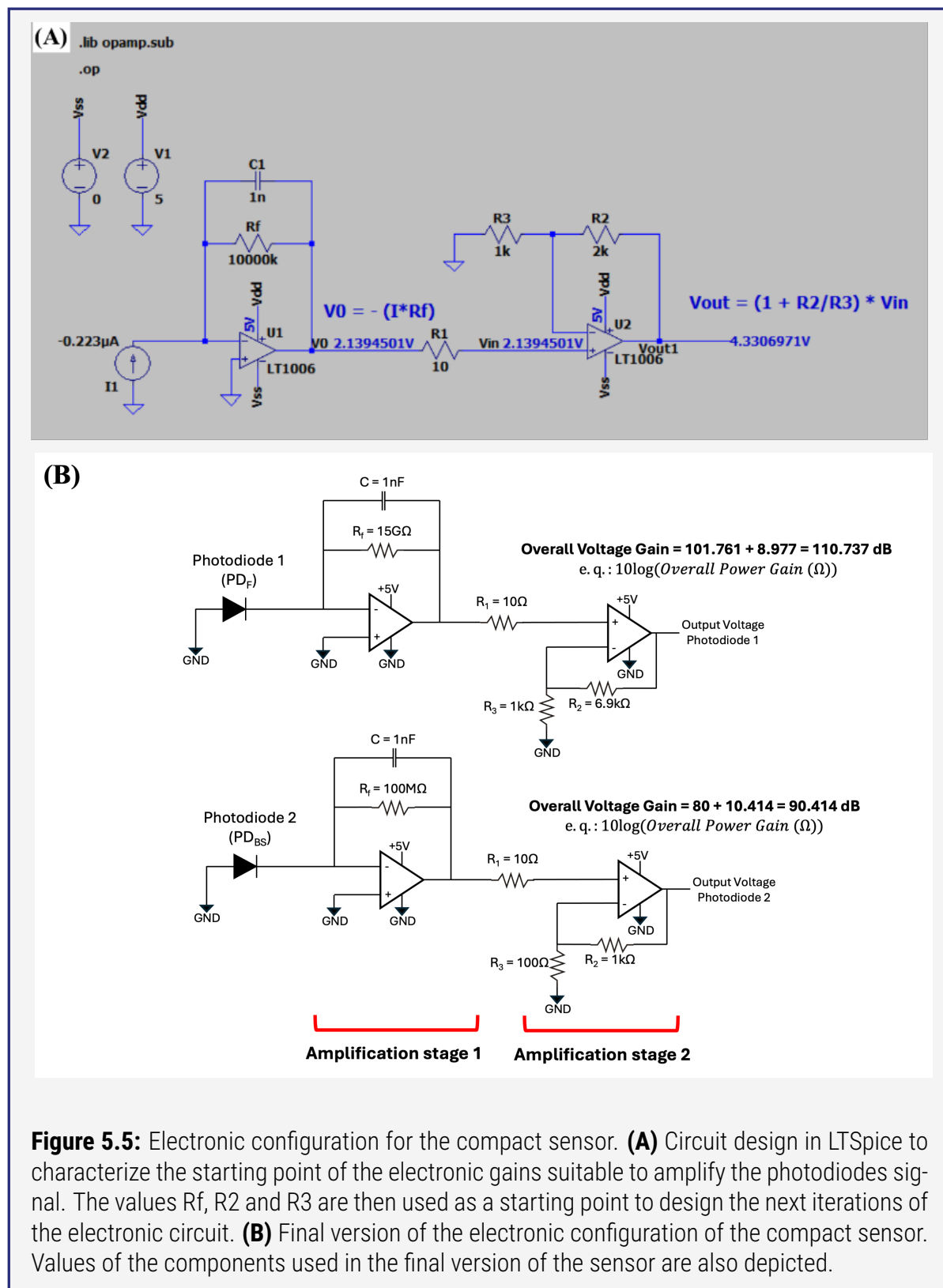
The primary focus of the electronic circuit is the amplification of the small fluorescence signal. The small current generated by the fluorescence signal (0.223 μA), and therefore, the need for particularly high electronic gains, influenced the use of amplifiers with specific requirements.

Usually, for most of the applications, the input bias current of an amplifier is negligible. However, in applications with significant small input currents ($< 1\mu\text{A}$), an accurate output is required and, therefore, the input bias current of the amplifier become a critical parameter to consider when selecting an amplifier. The input bias current can introduce an error in the amplifier output voltage and, due to the high amplification gain required for this application, it can significantly affect the collected signal.

In addition, because of the considerable amplification gain needed in the circuit, it is also important to maximize the dynamic range of the amplifiers. This is paramount when deploying the compact sensor in clinical studies with unknown patient variability (i.e., uncertain input and output voltage ranges). Therefore, in this case, a Rail-to-rail capability will facilitate the use of a bigger range of output Photodiodes voltages.

Power consumption is not contemplated in this case, as the sensor is powered through a USB cable and additional requirements, such as responsiveness, are not considered a priority. However, low-bandwidth amplifiers, generally, have a better noise performance and can introduce an advantage

for applications, such as in this case, with low input current and where high-speed response is not crucial.



Therefore, considering all the requirements presented above, the LMP7721 Amplifier (LMP7721 3-

Femtoampere Input Bias Current Precision Amplifier, Texas Instruments), with ultra-low input bias (3 fA), low offset voltage (150 μ V) and wide-gain bandwidth (17 MHz), was used in the first stage (represented in Fig. 5.5) to amplify the signals from the photodiodes. For the second amplification stage, with considerably smaller gain and less strict requirements, the MPC6002 amplifier (MPC6002, 1 MHz, Microchip) was used. Figure 5.5B shows the final electronic configuration and the values of the components used, including the first and second amplification stages for both photodiodes.

Continuing with the electronic characterization, to ensure thermal stability and safe use, a 70% duty cycle (with a 10 s time period) was applied to the LED (i.e. 7 s on, 3 s off), which was used throughout all measurements. This duty cycle was selected based on the characteristics of the detected fluorescence and backscattered signals. As shown in Fig. 5.4B, there is a time desynchronization between the Fluorescence and Backscatter Photodiodes. The small currents generated from the fluorescence signal and the corresponding high gain used lead to a delay in the Fluorescence Photodiode activation response (visible at around 0.5 s; Fig. 5.4B) as well as a slower increase towards a stationary state (plateau stage; Fig. 5.4B). This is due to slow charging of the photodiode's capacitor at the μ A current levels generated by the fluorescence signal and the slower amplifier response obtained when using high gain. Thus, a 70% duty cycle was selected – with the LED on for 7 s and off for 3 s – to ensure that the plateau stage was reached in both photodiodes. The plateau regions were then used to extract photodiode intensity values for calculation of the normalized fluorescence intensity (see Section 5.2.2.4).

This duty cycle also ensured that the LED temperature remained constant (thereby avoiding overheating) for the duration of measurements (i.e. when switched on for up to 3 hours) and allowed for measurement of normalized transcutaneous fluorescence intensity every 20 s. (An additional wait time of 10 s was incorporated into the LabView control software to allow for processing and to ensure that measurements were made at regular, repeatable intervals. This additional time also allows for an easier comparison with the Benchtop system.

Finally, as discussed above, the Backscatter Photodiode is used to correct for variations due to parameters such probe orientation/location, LED excitation power, and skin colour/thickness. As an example, Fig. 5.4A shows a decrease in the signals from both photodiodes at a time of approx. 77 minutes caused by a change in sensor position. As this is detectable by both photodiodes, the normalized fluorescence signal can be corrected as it is calculated by dividing the output voltage from the Fluorescence Photodiode by that from the Backscatter Photodiode – see Section 5.2.2.4.

5.2.2.4 Calculation of normalized fluorescence values

To account for signal variations due to factors such as excitation power fluctuations, probe orientation and skin tone, the collected fluorescence data is normalized according to the intensity of the backscattered excitation signal. This normalization was performed for data collected with both

the compact sensor and the control system (benchtop system – Section 5.2.1).

For the benchtop system, the normalization process was conducted as described in Section 5.2.1 and Ref. [90]. In the case of the compact sensor, it follows a similar procedure as the benchtop system but rather than working with integrated spectrum per data point, we work with single voltage values. Therefore, a pre-processing step was implemented in the software of the sensor to first subtract the baseline from the raw data collected in each LED duty cycle (for both the Fluorescence and Backscatter Photodiodes). This was achieved by calculating the average intensity value over a period when the LED was switched off (i.e., during the OFF window shown in Fig. 5.4B) and subtracting this value from the raw data at each time point. Fluorescence and backscattered intensity values were then calculated (based on the baseline-subtracted signals) by taking the average photodiode signals over a period when the LED was switched on and the observed signals had reached a plateau (i.e., during the ON window shown in Fig. 5.4B). A normalized fluorescence intensity value was then calculated for each LED duty cycle according to equation (5.2), where I_{norm} is the final normalized fluorescence intensity, I_F and I_L are the average fluorescence and backscattered intensity values respectively (i.e., values from the ON window in Fig. 5.4B), and I_{BGF} and I_{BGL} are the average background fluorescence and backscattered intensity respectively (i.e., values from the OFF window in Fig. 5.4B).

$$I_{norm} = \frac{I_F - I_{BGF}}{I_L - I_{BGL}} \quad (5.2)$$

As explained above, this normalization procedure generated fluorescence intensity values for the compact sensor and benchtop system that were internally corrected to account for variations in excitation power, probe orientation and other factors. Nonetheless, the absolute values produced in each case were different across the two devices (due to differences in the detection electronics used and the corresponding outputs – i.e., the photodiodes in the compact sensor provided voltage output values while the spectrometer in the benchtop system provided intensity measurements in arbitrary spectral units). To allow comparison of the datasets collected with each system, the collected fluorescence vs. time curves were scaled to their maximum intensity. This meant that the collected fluorescence vs. time curves had an intensity range of 0–1 for both the compact sensor and benchtop system, thereby allowing comparison of the datasets and investigation of the degree of correlation. Importantly, 0-1 scaling was only applied when comparing devices (i.e., in experimental procedure in Section 5.2.3.2 – Stage 1) and not when investigating the impact of dose and skin tone (i.e., Section 5.2.3.2 – Stages 2 and 3).

5.2.2.5 In-house written Control Software

The operation of the sensor, including data collection and pre-processing, is exclusively controlled with the developed LabView software. Initially, the power of the LED is limited and fixed through the electronic configuration, employing the use of an LED driver (as described in Section 5.2.2.3). Be-

sides this, the rest of the sensor, including LED duty cycle, is controlled by software written in-house using LabVIEW. The software includes four sections: sensor control (including LED triggering), logging settings, data visualization and input data control. A screenshot of the user interface is shown in Fig. 5.6.



Figure 5.6: The LabView user interface developed for collection and pre-processing of data. **(A)** Main tab of the LabView interface showing the raw voltage data collected by the sensor photodiodes (upper graph; PD0 – Backscatter Photodiode; PD1 – Fluorescence Photodiode), the normalized fluorescence intensity data (lower graph), and the main sensor/acquisition controls. **(B)** Secondary interface tab with additional information (including mean 'Off' and 'On' data for each photodiode).

The *Sensor Control* section includes settings regarding the input and output channels of the USB-

DAQ device. The Triggering Setting, within this section, controls the LED duty cycle, which the user can easily adjust by changing the time, in seconds, that the LED is ON (High Time) and OFF (Low Time). In the Logging Settings section below, the users also have the option to decide whether the data is saved (Log and Read) or not (Not saved). When the data is saved, three different files are created inside the folder that the user has previously determined. Data directly collected from the photodiodes (raw data) is saved in a TDMS file showing the fluorescence and a backscattered signals as a function of time, the pre-processed data (including the normalized data) is stored in a TXT file for easy accessibility and further analysis and, finally, an additional TXT file is created for any extra input information entered by the user through the “*Comment*” section.

The raw data, as well as the pre-processed normalized data, are displayed in the main tab of the interface (*User Tab*). Additional information, such as ON and Off mean data extracted from the raw data, is displayed in a secondary menu, accessible to the user on the “*Additional Information*” Tab.

Lastly, the *Input data* section allows the user to record the time point when the participant starts drinking the dye (*Drink button*) or any other custom events such as toilet breaks. All this information is accompanied with a description that the user can include inside the “*Comment*” section for extra clarifications.

Altogether, it demonstrates that the interface was created to automate data collection and to provide full control of the sensor that can easily be operated by a single operator.

5.2.2.6 Safety considerations

To ensure safe use in clinical experiments, the optical power and temperature of the LED were analysed using an optical power meter (PM100D, Thorlabs) and a thermocouple (MAX31855, Maxim Integrated Products) respectively. Measurements were performed at both the surface of the LED and at the distal tip of the fibre-optic probe, in separate days, for a period of 3 hours to investigate the stability of the sensor over the timescale of a typical experiment.

The optical power of the LED at the distal tip of the fibre probe was limited to a maximum allowable power of 63µW. This maximum allowable power (P_{max}) was calculated using the following equation and considering that the distal tip of the fibre probe is placed in contact with the skin (spacing $\ll 1\text{mm}$):

$$\text{Area}_{LED-distal\ fibretip} = \pi * r^2 = \pi * (100 * 10^{-6})^2 = 10^{-8} \pi m^2 \quad (5.3)$$

The Maximum permissible exposure (MPE) for skin is given by [150]:

$$MPE = 2 * C_A \quad (5.4)$$

where C_A is the correction factor equal to 1 for the LED wavelength (465 nm) [150]. The P_{max} at LED-skin distance $\ll 1$ mm (LED touching the skin) for skin-safe use is:

$$P_{max} = MPE * Area_{LED} \quad (5.5)$$

$$P_{max} = (2 * 1) * 10^{-8} \pi = 2\pi * 10^{-8} kW \approx 63 \mu W \quad (5.6)$$

A constant current LED driver limited the LED current to 63 μA to ensure the light emitted at the distal end of the fibre-optic probe was always below the maximum permissible exposure (MPE) for the skin and to mimic the optical power of the control system used for the validation (see Section 5.2.1)

5.2.3 Experimental procedures - validation

5.2.3.1 Fluorescence samples measurement – laboratory validation

We first evaluated the sensitivity of the compact fluorescence sensor for detection of different concentrations of fluorescein in aqueous solution. A series of fluorescent samples (fluorescein solutions) were prepared for this purpose, designed to approximate the fluorescence intensities observed in vivo (based on previous data collected using the benchtop system in healthy volunteers). We note that these fluorescent samples were not intended to act as realistic phantoms. Rather, they simply served to provide specific fluorescence intensity levels that corresponded to those observed in vivo, thereby allowing us to determine whether the sensitivity of the compact sensor was sufficient to detect transcutaneous fluorescein fluorescence. Thus, fluorescein solutions were prepared in water at concentrations of 0 mg/ml (i.e., water with no fluorescein which represents the baseline), 0.00044 mg/ml, 0.00056 mg/ml, 0.00083 mg/ml and 0.0017 mg/ml. The fluorescein solutions were contained within transparent thermoplastic pouches for measurements, and fluorescent signals were evaluated using both the compact fluorescence sensor and the benchtop control system (experimental set up is shown in Fig. 5.7). The transparent containers were built using thermoplastic material (polyethylene (PE) polyethylene terephthalate (PET) triple laminate, PE/PET/PE, of thickness 30/60/30 μm) and designed using laser welding technique, previously presented in Ref. [232], in which a laser beam selectively welds thermoplastic sheet materials together following a pre-defined two-dimensional pattern. Once the process is finished, the pre-defined pattern, designed in SolidWorks, creates a 3D cylindrical container that can hold approximately 25 ml of fluorescence solution.

It is important to mention that movement of the arm can potentially affect the readings, as the sensor is directly attached to the fingertip of the participants using a 3D printed finger clip. Therefore, further in vitro validation of the sensor was focused on analysing the sensitivity of the sensor to mo-

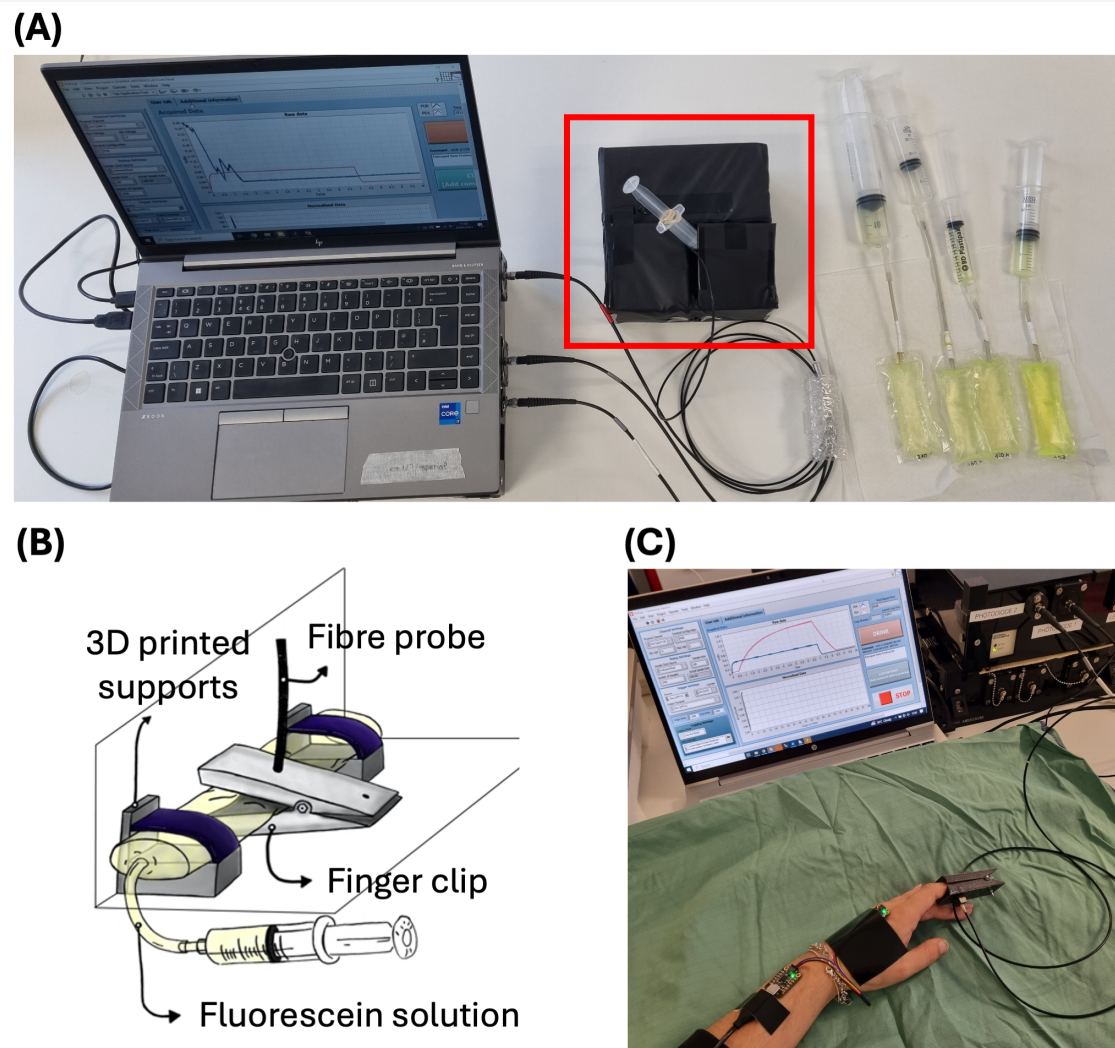


Figure 5.7: *In vitro* experimental setup. **(A)** Photograph of experimental setup. The sensor (placed below the laptop) collects the fluorescence intensity of fluorescein solutions in transparent thermoplastic containers. Thermoplastic containers are displayed on the right-hand side of the image and contain aqueous fluorescein solutions at concentrations of 0 0.0017 mg/ml. Container with 0 mg/ml fluorescein (i.e., baseline containing only water) is not visible in the image. This container is located inside a black box (marked in red), which was used to minimize background signals. **(B)** Diagram of the inside of the black box (marked in red in panel A) used to collect fluorescence data. The fluorescein solution is attached to the inside of the box using 3D printed supports to maintain a constant position during measurements. The finger clip (including the optical fibre probe) is clamped to the fluorescein sample. The spring-loaded mechanism of the finger clip allows a secure and constant position to be maintained throughout the collection time. **(C)** Experimental set up for Motion artifact analysis. The sensor is attached to the fingertip of one of the participants and an accelerator is secured on top of the same hand. The sensor and the accelerometer were subjected to three hand-motion scenarios: static (no motion), low and fast movements. The accelerometer collected data from the x, y and z-axes.

tion artifacts using an accelerometer (Adafruit MSA311 Triple Axis Accelerometer – STEMMA QT / Qwiic) as the key investigative tool. The sensor, attached to the fingertip of the participant using the 3D printed finger clip, was constantly recording data, together with an accelerometer placed on the top of the same hand to capture synchronized data (set-up is shown in Fig. 5.7C). Three different levels of motion: static, slow movements and fast movements were recorded. Datasets from both the sensor and the accelerometer were synchronized to evaluate the impact of motion artifacts on the developed sensor accuracy.

5.2.3.2 Human measurement – in vivo validation

In vivo validation of the compact fluorescence sensor was performed in three stages. First, clinical measurements were undertaken in 8 healthy volunteers using both devices (i.e., the benchtop system and the compact fluorescence sensor) simultaneously to compare the collected signals (Stage 1). Second, additional measurements (using both devices) were performed in 6 further healthy volunteers using a lower fluorescein dose to demonstrate the potential of the sensor to assess gut function under different dose conditions (Stage 2). Third, all 14 healthy volunteers were classified into three groups based on their skin tone (classified according to the Fitzpatrick scale [233]) to determine the performance of the sensor on different skin tones (Stage 3). For all measurements, the benchtop was attached to the participant's index finger and the compact sensor to the participant's middle finger. The information about participant demographics, as well as the fluorescein dose used in each participant, is shown in Table 5.1.

All volunteers gave informed consent prior to the experiments. All the 14 volunteers' experiments were performed at St. Mary's Hospital (London, UK) according to a local clinical study protocol [23] (details of ethical approval: UK Health Research Authority IRAS Project ID – 242462; Research Ethics Committee reference – 18/LO/0714/AM04). All experiments were performed in accordance with Good Clinical Practice (GCP) guidelines and the World Medical Association's Declaration of Helsinki.

For **Stage 1**, the fibre probes of the compact fluorescence sensor and the benchtop system were first attached to the participant's first and middle fingers respectively. Fluorescence measurements were then started at the same time the participant was asked to begin drinking a fluorescein solution containing 500 mg fluorescein dissolved in 100 ml water. Fluorescence signals (from the compact sensor) and fluorescence spectra (from the benchtop system) were then recorded for a total of 180 minutes (with the exception of participants 1 and 2, for whom the experiments were terminated early – after 90 and 135 minutes respectively), with normalized fluorescence intensity values (see 5.2.2.4) calculated and reported every 60s and 20s for the benchtop system and compact sensor respectively.

For **Stage 2**, both devices (i.e. the benchtop system and the compact fluorescence sensor) were again used simultaneously, this time with a lower dose of fluorescent dye. The fibre-optic probes

were attached to the participant's fingers, and participants were asked to drink a fluorescein solution containing 200 mg of fluorescein dissolved in 100 ml water (as opposed to 500 mg fluorescein used in Stage 1). Fluorescence signals were recorded as in Stage 1.

For **Stage 3**, all 14 participants were classified into three groups based on their Fitzpatrick skin type. No extra data was collected for this stage. Instead, Stage 3 focused on analysing the collected data to assess the impact of skin tone on the performance of the sensor. First, we analysed the optical baseline signals (extracted from the first 10 minutes of data) where no fluorescence signal from the contrast agent is present. Second, we analysed the entire fluorescence vs. time curves (i.e., 180 minutes). In all experimental stages, all volunteers were seated for the duration of the experiments and were asked to fast overnight prior to the experiments.

Table 5.1: Demographics of the study participants and the respective ingested dose of fluorescent dye. *Stage 1:* Participants 1-8; fluorescein dose – 500 mg (in 100 ml water). *Stage 2:* Participants 9-14; fluorescein dose – 200 mg (in 100 ml water). Lower region of the table presents the percentages and numbers (in brackets) of participants in each skin tone and dose group.

ID	Sex	Age	Fitzpatrick score	Fluorescence Dose
P1	F	24	3	500mg
P2	F	24	1	500mg
P3	F	24	1	500mg
P4	M	23	3	500mg
P5	F	23	4	500mg
P6	M	23	3	500mg
P7	M	21	5	500mg
P8	M	56	3	500mg
P9	F	24	1	200mg
P10	M	27	3	200mg
P11	F	23	1	200mg
P12	M	37	5	200mg
P13	F	21	3	200mg
P14	M	23	3	200mg
Total Participants				14
Male / Female				50%/50%
Fitzpatrick class 1 and 2				29% (4)
Fitzpatrick class 3 and 4				50% (7)
Fitzpatrick class 5 and 6				21% (3)
Participants with 500mg dose				57% (8)
Participants with 200mg dose				43% (6)

5.2.4 Data Analysis

When analysing the fluorescence data, one must consider the fact that the data is affected by variation in the optical power transmitted by the probe, but also by motion artifacts. Thus, the use of the Backscattering Photodiode and the Data Normalization process (explained in Sections 5.2.2.3 and 5.2.2.4), mitigate the effects produced by variations in the LED power, as well as changes in the detection area caused by motion artifacts. In addition, the post-processing of the data also involves an initial (1-D median) filtering step to remove any unwanted noise or interferences.

Statistical tests were performed to analyse correlation/differences between devices (*Stage 1*) and to assess the performance of the compact sensor under different experimental conditions (*Stages 2 and 3*). Pearson's correlation coefficient (r) was calculated to measure the linear correlation between datasets collected with the compact fluorescence sensor and benchtop system (using the scaled fluorescence signals for both individual and mean fluorescence vs. time curves). P -values < 0.05 were used to infer statistically significant correlations. Pearson's correlation coefficients (r) were calculated using the "*corrcoef*" MATLAB function.

Student t-tests were used to analyse differences between data collected with each device (*Stage 1, paired T-test*) and data collected under different dose and skin tone conditions (*Stages 2 and 3, unpaired T-test*). For this purpose, the Peak intensity, Peak time, Total area under the curve (AUC) and area under the curve up until the time of peak (Peak AUC) were extracted from each dataset and compared across groups (with scaled fluorescence signals (0 - 1) used for *Stage 1* and normalized fluorescence signals used for *Stages 2 and 3*). As explained in Chapter 4, these parameters were chosen to provide a simple characterisation of the shape and intensity of the fluorescence vs time curves collected with the two devices. The total AUC, for example, provides a characterisation of the total fluorescence intensity, whereas the peak time provides an indication of the rate of the uptake of fluorescein. Thus, these parameters served to allow an effective assessment and comparison of the data collected with different devices and under different conditions.

5.3 Results

5.3.1 Laboratory validation

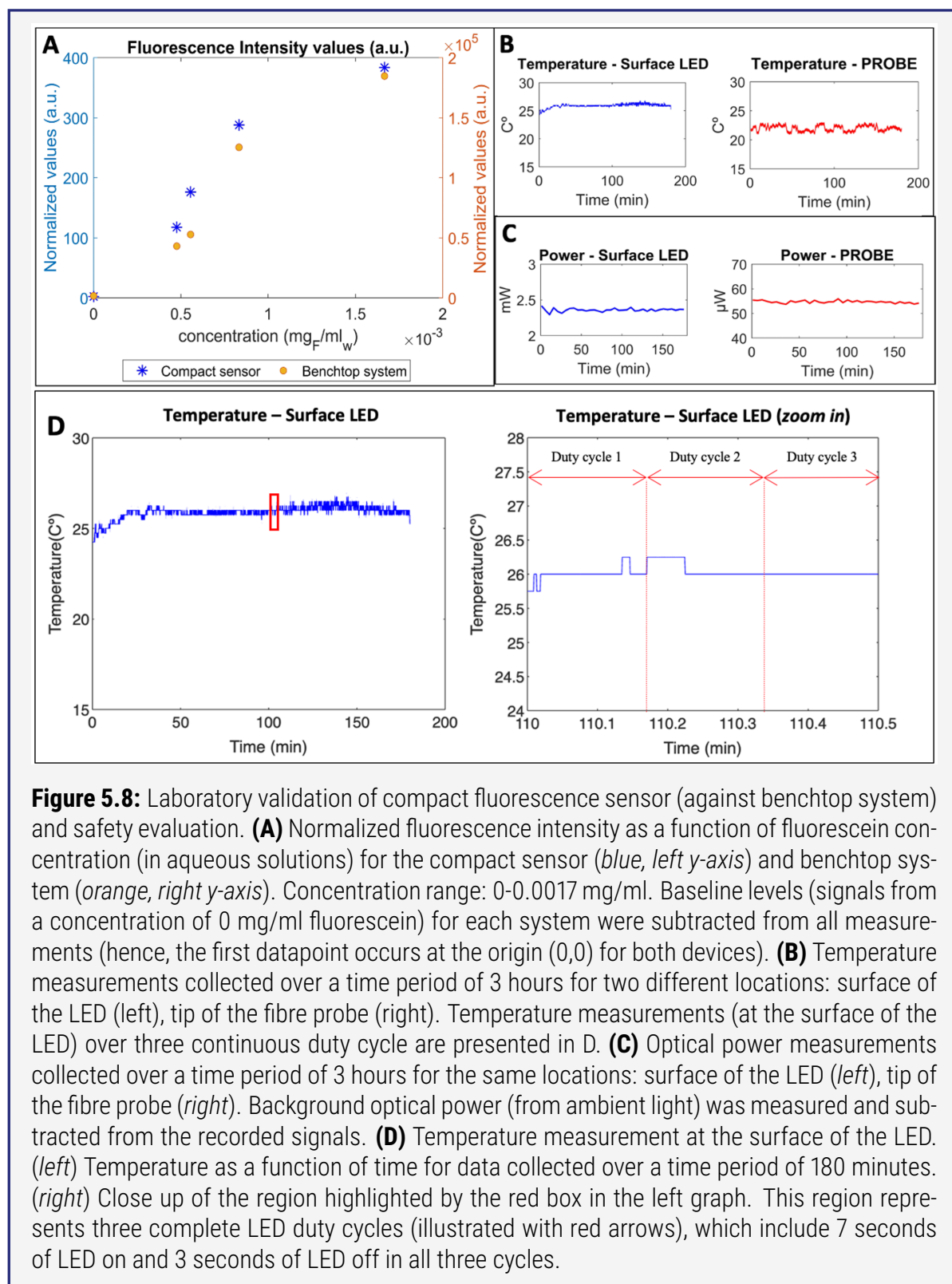
5.3.1.1 Fluorescent samples measurements

To validate the compact fluorescence sensor, we first assessed the capability to detect fluorescein fluorescence in aqueous solutions with different known dye concentrations. The fluorescein concentrations used were specifically selected to approximate transcutaneous signal levels expected in healthy participants. To this end, the fluorescein concentrations were calculated based on transcutaneous data previously collected from healthy participants (i.e. as reported in [90]). Participants drank a solution of 500 mg fluorescein in 100 mg water and the benchtop system recorded the fluorescence signals for 3 hours. The peak intensities observed in those transcutaneous datasets were then compared against fluorescence signals collected (using the benchtop system) from a series of aqueous fluorescein solutions with known concentrations. Based on these measurements, we were able to pair the transcutaneous signal with an approximate corresponding dye concentration in aqueous solution. The chosen aqueous fluorescein concentrations for onward experiments were thus in the range 0 – 0.0017 mg/ml, as this range was found to approximate the varying level of fluorescein fluorescence detected during transcutaneous experiments.

Laser welded [232] thermoplastic containers were fabricated and filled with the chosen fluorescein solutions. A non-fluorescent solution (i.e., water; dye concentration = 0 mg/ml) was also measured to assess any background signals (e.g., from the encapsulation material).

Following preparation of the fluorescein solutions, fluorescence measurements were performed using both the compact sensor and benchtop system. Fluorescence spectra peak intensities from the benchtop system were compared against output voltages from the compact sensor, and similar trends (with respect to fluorescein concentration) were observed for both devices (Fig. 5.8A). A Pearson's correlation coefficient of $r = 0.983$ was obtained when comparing the two datasets, with a p -value of 0.003. In addition, a linear regression model (i.e., for the equation $y = mx + c$ (where m represents the gradient and c represents the intercept) was fit to both datasets. For the benchtop data, the linear regression yielded $R^2 = 0.944$ (p value = 0.005) with $m = 1.15 * 10^8 (mg/ml)^{-1}$ (units = $\left[\frac{\text{normalised fluorescence intensity}}{\text{fluorescence concentration}} \right]$) and $c = 178$ a.u. (units = [normalised fluorescence intensity]). For the compact sensor data, the linear regression model yielded $m = 2.31 * 10^5 (mg/ml)^{-1}$ and $c = 30.3$ a.u., with an R^2 value of 0.922 (p value = 0.009). These results demonstrate strong (statistically significant) linear correlations between the fluorescence intensity and fluorescein concentrations for both the benchtop and compact sensors.

Taken together, these observations suggested that the compact fluorescence sensor was capable of detecting fluorescein fluorescence with signal levels that would be expected *in vivo*.



5.3.1.2 Temperature and optical power measurements

To confirm the compact fluorescence sensor was safe for use in clinical experiments, temperature and optical power measurements were performed for 3 hours (i.e., the duration of a typical clinical experiment) using a thermocouple and an optical power meter. Two locations were investigated: the surface of the LED and the tip of the fiber probe. The temperature was stable over time at both locations (Fig. 5.8B) and was also stable over individual LED duty cycles (Fig. 5.8D). Furthermore, the average temperatures measured at the LED and the fibre tip (25.9 C° and 22.0 C° respectively) were found to lie within a safe range (i.e., close to room temperature), indicating that the compact sensor would not expose participants to unpleasant or dangerous temperature conditions.

Similarly, the optical power exhibited only minor variations over the duration of the experiment (at both the LED surface and fibre tip; see Fig. 5.8C), indicating stability of the light source current provided by the LED driver. The average optical power at the tip of the fibre probe was measured as 54.7 μW , below the maximum allowable power of 63 μW (which was calculated according to the guidelines presented in [150]).

5.3.1.3 Motion Artifact analysis

Further analysis evaluated the impact of motion artifacts on the accuracy of the compact sensor. To do so, an accelerometer, attached to the same hand as the compact sensor, collected data regarding hand motion during a period of 50 minutes. Datasets from both, sensor and accelerometer, were then synchronized to identify disturbances in the output signal of the sensor attributed to hand-motion artifacts.

Figure 5.9 shows the data simultaneously collected from the compact fluorescence sensor and the accelerometer for 50 minutes from one of the participants. The three testing motion scenarios: static, low movements and fast movements are visually identified in the accelerometer dataset. The static stage is characterized by the absence of displacement in all three axes (x, y and z), whereas slow and fast movement stages exhibit a progressive increase in displacements with the rising frequency of hand movements. As for the compact sensor, no perturbations or significant variations are manifested in the data for any of the hand-motion patterns. These observations suggest that the spring-loaded mechanism on the finger clip ensures a secure attachment and a consistent contact between the fibre probe and the skin, reducing the risk of signal disturbances caused by motion artifacts.

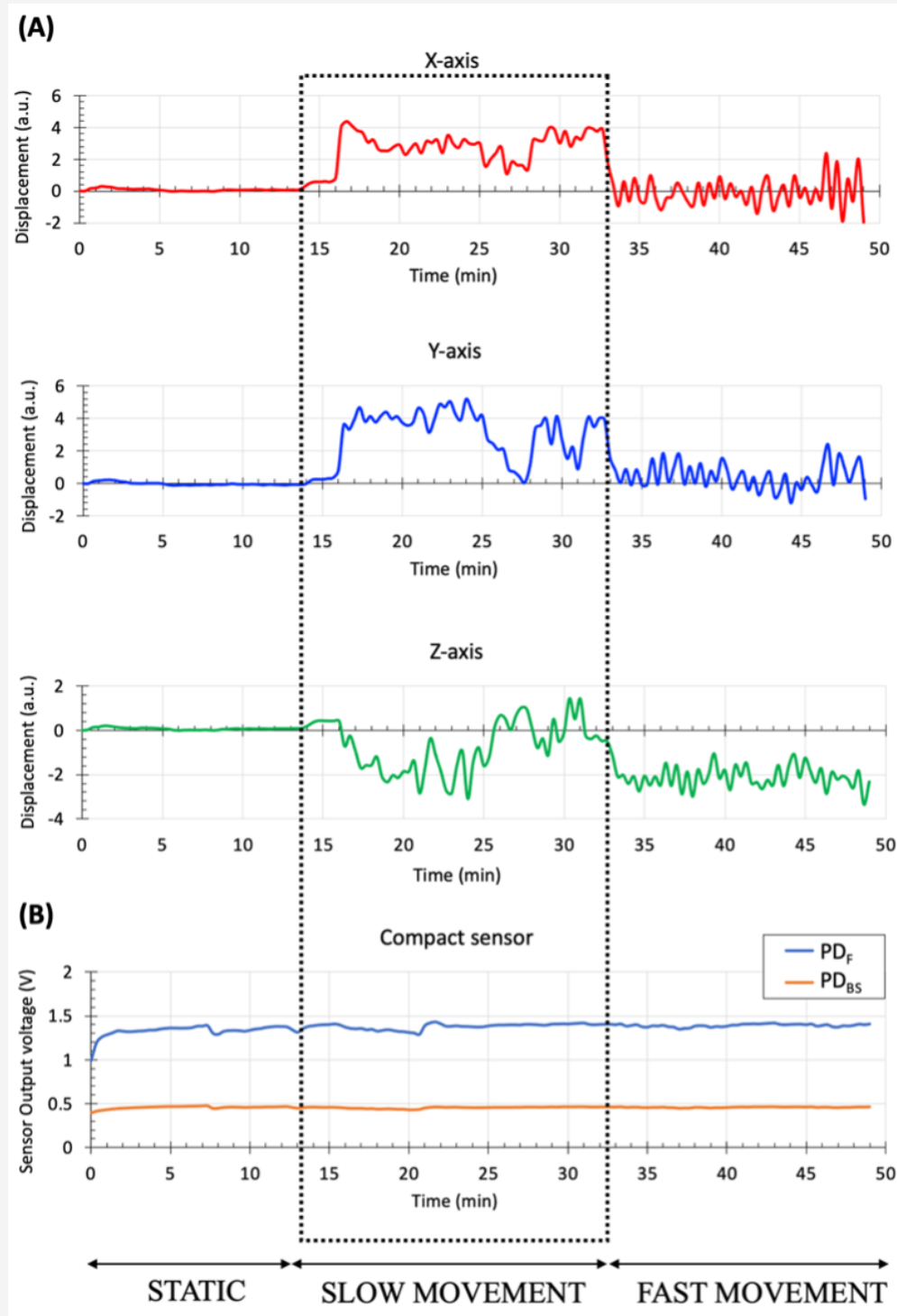


Figure 5.9: Motion Artifact analysis: comparison of different hand-motion scenarios and compact sensor stability for a period of 50 minutes. **(A)** Displacement along the x, y and z axes derived from the accelerometer dataset. The three motion scenarios: static, slow and fast movements, are visually identifiable, as the frequency and displacement patterns differ between scenarios. **(B)** Sensor output voltages from both Photodiodes (PD_F : Fluorescence Photodiodes and PD_{BS} : Backscattered Photodiodes).

5.3.2 In vivo validation

5.3.2.1 Fluorescence data Analysis – Stage 1

To investigate the potential of the compact fluorescence sensor as a portable tool for non-invasive assessment of gut function using transcutaneous fluorescence spectroscopy, we compared the performance against an existing benchtop system (presented in Ref. [90, 145, 209]). To do so, 8 healthy volunteers (participants 1-8) were recruited for an in vivo validation experiment in which the compact sensor and benchtop system were used to record fluorescence profiles simultaneously. As the benchtop system has previously been shown to be suitable for non-invasive sensing of both gut permeability [90, 145, 209] and gastric emptying rate [145], validation against this system served to demonstrate the potential of the compact sensor for the same purpose with the advantages of a smaller footprint and lower cost.

Eight participants were asked to fast for a minimum of 5 hours prior to the experiment. At the start of the measurement, the fibre-optic probes from the compact sensor and the benchtop system were attached to the participant's fingers using 3D-printed finger clips. Participants were asked to drink a fluorescein solution (500 mg fluorescein dissolved in 100 ml water), and fluorescence data was collected with both devices for 180 minutes. Fluorescence intensity vs. time curves (scaled from 0-1) from both systems were plotted to compare the two datasets (Fig. 5.10). As expected, fluorescence intensity was observed to increase over time, reaching a peak value approximately 40 minutes after ingestion of the fluorescein solution, before beginning to decrease back towards the background level (Fig. 5.10). We note that small differences in the decay rate were observed between the benchtop system and the compact sensor in some participants (e.g. see Participants 3, 6 and 8; Fig. 5.11). These can be tentatively attributed to: variations in vascular anatomy at the measurement sites (as the benchtop and compact systems were connected to different fingers); motion artifacts or probe movement; and/or probe placement differences. Despite these small discrepancies in the decay region, good qualitative agreement was nonetheless observed between the compact fluorescence sensor data and the benchtop data, in all 8 participants, both, individually (Fig. 5.10A and Fig. 5.11) and in the mean fluorescence vs. time curves (Fig. 5.10B).

To quantify the correlation between the datasets, Pearson's correlation coefficients were calculated for all individual measurements (presented in Fig. 5.11) and the average curves (Fig. 5.10B). As shown in Table 5.2, all Pearson's correlation coefficients (r) exceed 0.919 (with p -values < 0.001 in all cases).

To further assess the similarity between the datasets, we compared the parameters extracted from all individual curves (Fig. 5.11) as well as the average fluorescence intensity curves for both systems (Fig. 5.10B). The parameters extracted from the individual curves are presented in Table 5.3. As shown in Fig. 5.10C, both datasets exhibited similar Total AUC values, with a difference of 6.787% (which was not statistically significant, as determined by a paired Student t-test (p -value =

0.142). Total AUC values and other extracted parameters (such as Peak AUC and Peak Time) for all individual signals, as well as the statistical analysis results, are presented in Tables 5.3 and 5.4. No significant differences (p values > 0.05) were found in any of the comparisons, as determined by a paired Student T-test.

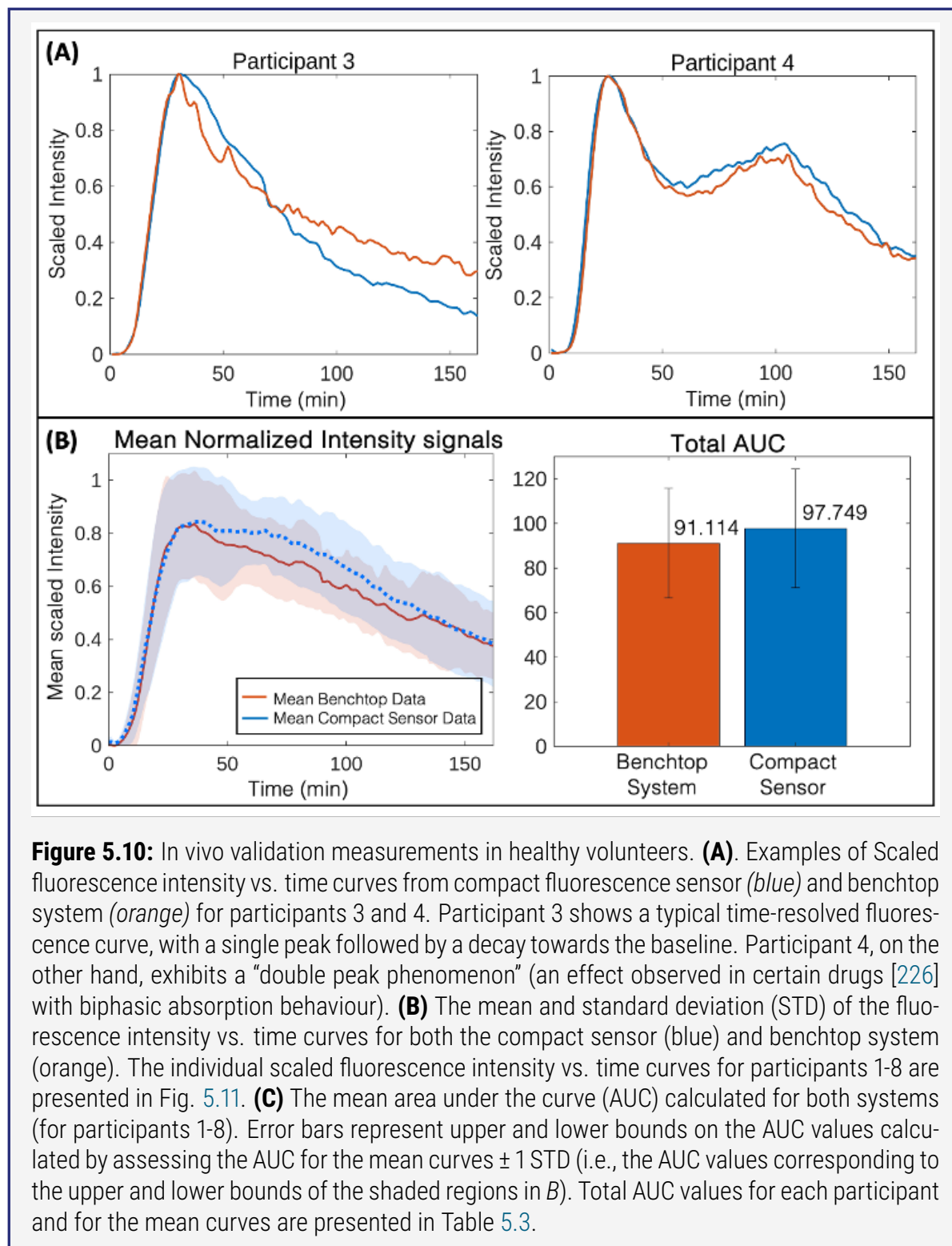


Figure 5.10: In vivo validation measurements in healthy volunteers. **(A)** Examples of Scaled fluorescence intensity vs. time curves from compact fluorescence sensor (*blue*) and benchtop system (*orange*) for participants 3 and 4. Participant 3 shows a typical time-resolved fluorescence curve, with a single peak followed by a decay towards the baseline. Participant 4, on the other hand, exhibits a “double peak phenomenon” (an effect observed in certain drugs [226] with biphasic absorption behaviour). **(B)** The mean and standard deviation (STD) of the fluorescence intensity vs. time curves for both the compact sensor (*blue*) and benchtop system (*orange*). The individual scaled fluorescence intensity vs. time curves for participants 1-8 are presented in Fig. 5.11. **(C)** The mean area under the curve (AUC) calculated for both systems (for participants 1-8). Error bars represent upper and lower bounds on the AUC values calculated by assessing the AUC for the mean curves ± 1 STD (i.e., the AUC values corresponding to the upper and lower bounds of the shaded regions in B). Total AUC values for each participant and for the mean curves are presented in Table 5.3.

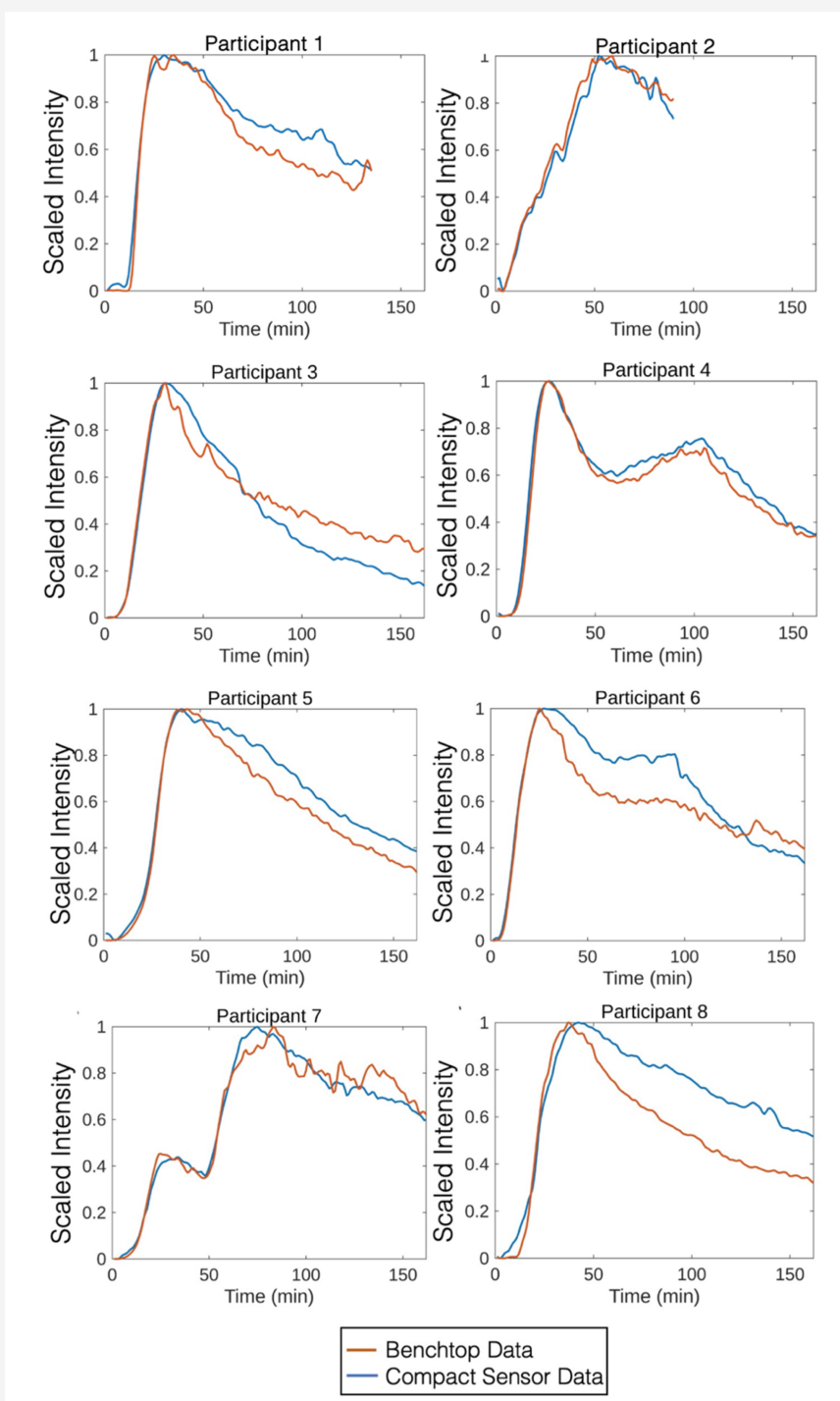


Figure 5.11: Individual scaled fluorescence intensity vs. time curves for participants 1-8. Benchtop system – orange; compact sensor – blue. Signals were recorded for a total of 180 minutes, with the exception of participants 1 and 2, where data collection issues necessitated early termination of experiments (after 135 and 90 minutes respectively). The presented fluorescence vs. time curves are scaled to their maximum intensity (from 0 – 1) in order to address the different absolute values produced by the compact sensor and benchtop system (due to differences in the detection electronics used and the corresponding outputs). This facilitated investigation of correlation between datasets collected with the two devices.

Table 5.2: Pearson's correlation coefficients and p-values for the datasets presented in Fig. 5.10 and Fig. 5.11.

ID	Correlation coefficient (r)	Correlation coefficient <i>p</i> value	95% confidence interval (lower and upper bounds)
P1	0.978	<0.001	[0.970 0.984]
P2	0.994	<0.001	[0.992 0.996]
P3	0.964	<0.001	[0.951 0.973]
P4	0.993	<0.001	[0.991 0.995]
P5	0.988	<0.001	[0.984 0.991]
P6	0.935	<0.001	[0.914 0.951]
P7	0.982	<0.001	[0.977 0.987]
P8	0.919	<0.001	[0.892 0.939]
Mean curves	0.989	<0.001	[0.986 0.992]

Overall, this demonstrated that the performance of the compact fluorescence sensor was comparable with the clinically validated Benchtop system, thereby indicating its potential for clinical assessment of gut function.

As an aside, it is worth noting that the range normalization approach used in this section (i.e., scaling data from 0 to 1) would not be suitable for investigation of intestinal permeability (where it is necessary to assess changes in fluorescence intensity between participants, e.g., as described in Ref. [209]). Instead, this approach served to allow comparison and validation of the compact sensor against an existing system that has been deployed in clinical studies, thereby demonstrating development of a more affordable and portable technology that is better suited to large-scale clinical deployment.

Table 5.3: Parameters extracted from the datasets presented in Fig. 5.10 and Fig. 5.11

AUC: area under curve. Peak AUC represents the AUC value calculated from time = 0 minutes until the time at which maximum intensity is observed (Peak Time). To compare the extracted parameters, signals from each participant and system were first scaled to their maximum intensity (i.e. range = 0–1). Parameters for participants 3-8 were extracted from the full 180 minutes of data. Parameters for participants 1 and 2 were extracted from 135 and 90 minutes of data respectively (due to the early termination of data collection explained above). The Table includes Peak Time, Total AUC and Peak AUC values for each participant. The same parameters were also extracted from the mean curves (displayed in Fig. 5.10B) and are shown at the end of the table (including values from error bars, which correspond to the upper and lower bounds of the shaded regions in Fig. 5.10B). Note that no STD values are included for Peak Time as time points for data collection were the same across all participants.

ID	System	Peak Time	Total AUC	Peak AUC
P1	Benchtop system	35	81.28	17.072
	Compact sensor	30	90.273	13.001
P2	Benchtop system	59	60.832	33.122
	Compact sensor	52	58.721	24.517
P3	Benchtop system	30	78.729	12.089
	Compact sensor	31	70.614	12.674
P4	Benchtop system	26	95.704	8.832
	Compact sensor	27	101.553	10.783
P5	Benchtop system	43	93.097	16.632
	Compact sensor	40	103.599	14.293
P6	Benchtop system	25	99.953	10.977
	Compact sensor	28	109.825	14.284
P7	Benchtop system	83	111.608	39.572
	Compact sensor	75	109.857	32.918
P8	Benchtop system	37	90.674	15.124
	Compact sensor	42	117.328	19.816
Mean curves	Benchtop system	37	91.114	15.807
	Compact sensor	39	97.749	17.755
STD	Benchtop system	-	24.594	3.055
	Compact sensor	-	26.559	2.818

Table 5.4: Statistical Analysis of both systems (Benchtop system and Compact sensor) using the parameters shown in Table . *P value* statistically significant < 0.05.

Parameter	Test	<i>p value</i>
Peak Time	Student T-test (paired)	0.371
Total AUC	Student T-test (paired)	0.142
Peak AUC	Student T-test (paired)	0.441

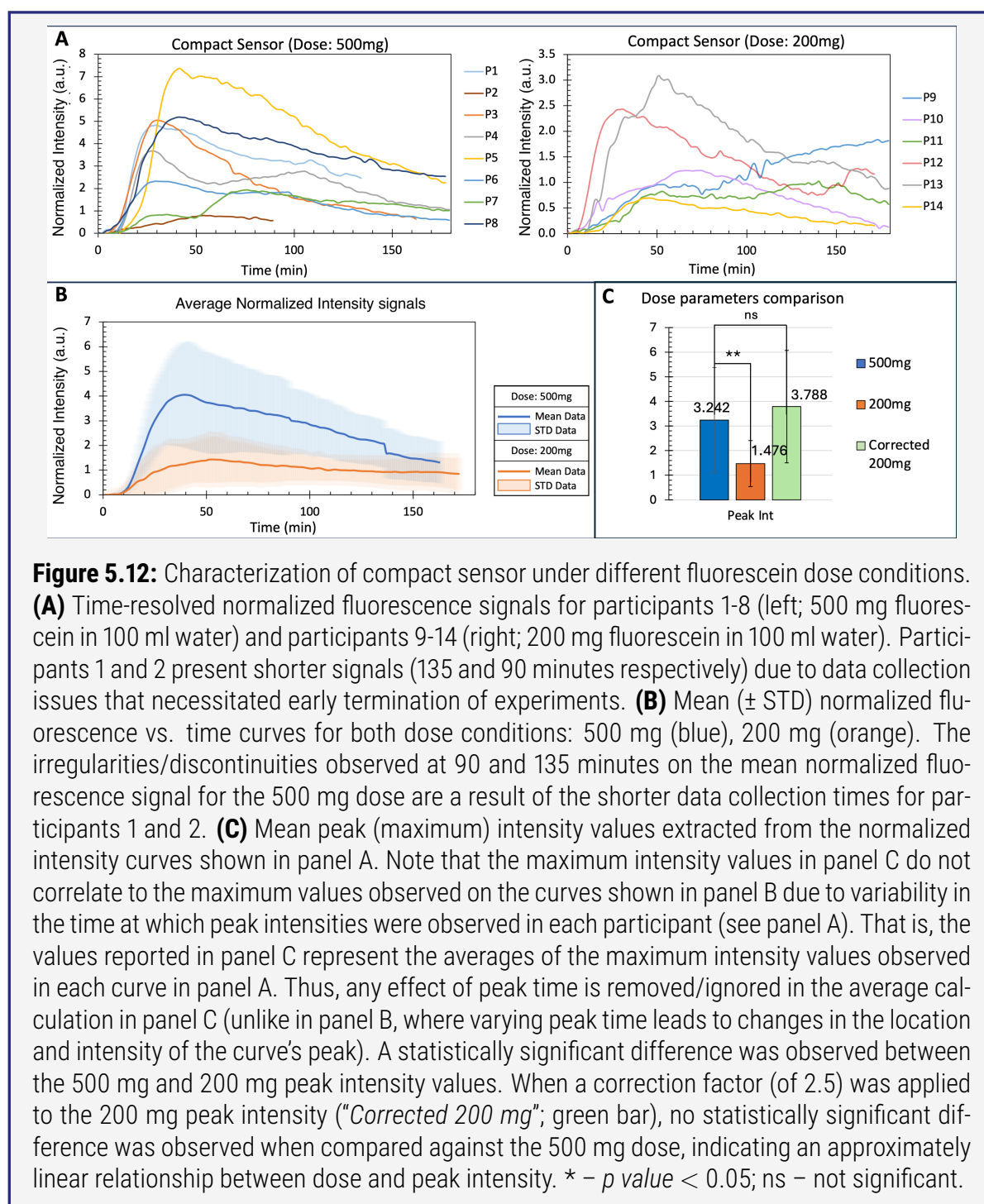
5.3.2.2 Compact sensor characterization – Impact of contrast agent dose – Stage 2

Following validation against the Benchtop device, a second experimental stage was introduced to characterize the response of the compact fluorescence sensor using a lower fluorescein dose. To do so, 6 further healthy volunteers (participants 9-14) were recruited following the same experimental protocol described above but with a lower fluorescein dose (200 mg fluorescein in 100 ml water).

Fig. 5.12A shows the fluorescence vs. time curves for all 14 participants (*left* – participants 1-8, 500 mg fluorescein; *right* – participants 9-14, 200 mg fluorescein). In this case, normalized fluorescence intensity signals are presented (as opposed to the scaled signals used in Sec. 5.3.2.1) to allow quantification of changes in fluorescence intensity with respect to dose. As observed in Fig. 5.12A, most of the datasets exhibit an increase in fluorescence intensity up to a peak point prior to a decay back towards zero. A similar trend is observed in the mean fluorescence intensity vs. time curves for both dose conditions (Fig. 5.12B).

The only exception to this trend is the data collected in Participant 9, in which no decay region is observed and where peak intensity appears to occur at approx. 180 minutes (i.e., at the end of the 3hour acquisition). Importantly, the dynamics observed for participant 9 were the same with both the compact sensor (Fig. 5.12A) and the benchtop system (Fig 5.14A). Thus, this behaviour can be attributed to physiological factors intrinsic to the participant (e.g., slow gastric emptying, low gastrointestinal motility, etc.).

The maximum fluorescence intensity observed for the 500 mg dose (4.053 a.u. from the average curves) was higher than that observed for the 200 mg dose (1.433 a.u.) by a factor of 2.8 (Fig. 5.12B and also see Fig. 5.13). This is also observed when calculating the peak intensity amongst all participants, as shown in Fig. 5.12C. This difference was found to be statistically significant using an unpaired Student's T-test (*p-value* = 0.0371). Importantly, this significant difference is compensated when a correction factor of 2.5 is applied to the 200 mg dose signals (*p-value* = 0.741), suggesting an approximately linear correlation between dose and fluorescence intensity (i.e., indicating that a 2.5-fold increase in dose produces an approx. 2.5-fold increase in intensity). Results obtained from analysis of the Total AUC and analysis of equivalent data collected with the



benchtop system (collected at the same time as the data presented in Fig. 5.11) further support this hypothesis (see Figs. 5.14, 5.15, 5.16).

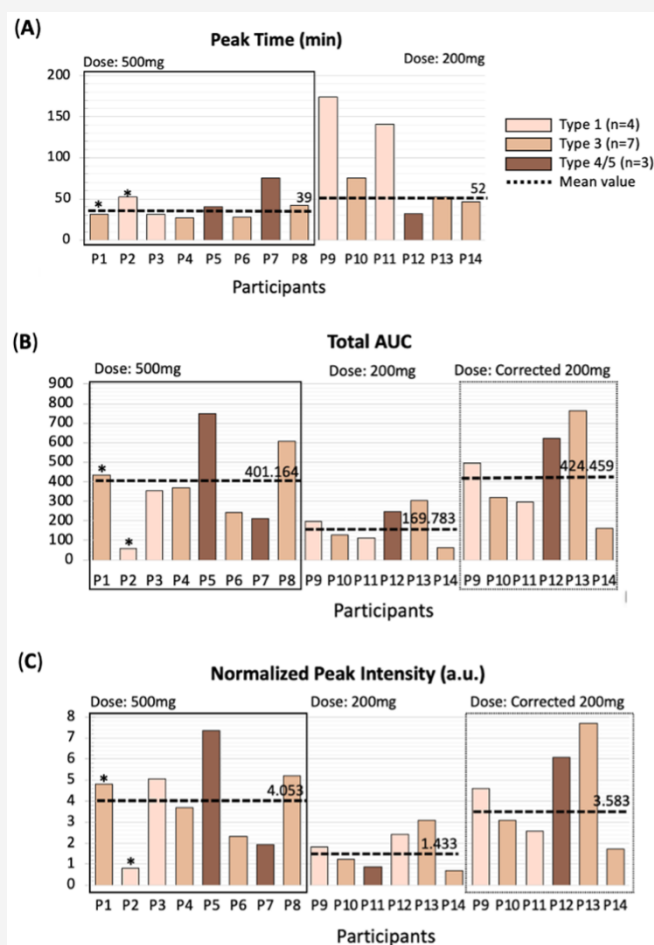
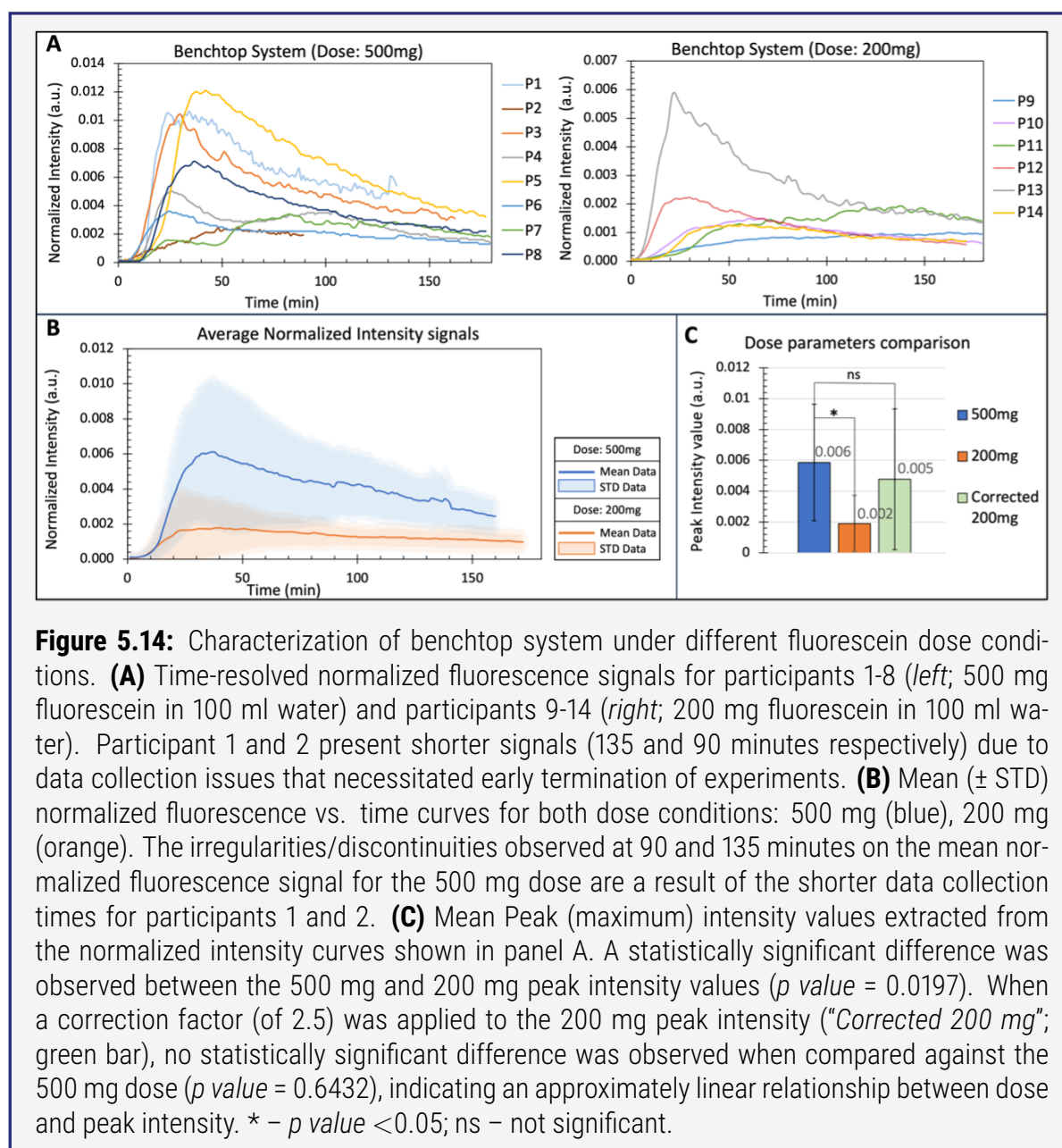


Figure 5.13: Peak Time, Total AUC and Normalized Peak Intensity values calculated from the compact sensor datasets for all participants (P1–14). Values for participants 3–14 were extracted from the full 180 minutes of collected data. Due to early termination of data collection (explained above), parameters for participants 1 and 2 (indicated with an *) were extracted from 135 and 90 minutes of data respectively. On each graph, data is classified according to contrast agent dose (including the “Corrected 200 mg” data, where data collected at 200 mg was multiplied by a correction factor of 2.5 to allow comparison to data collected at 500 mg). Bars are color-coded based on the Fitzpatrick skin tone type for each participant (see legend). The numbers of participants in each Fitzpatrick group are shown in the legend. Dotted lines represent the values from the mean fluorescence vs. time curves (presented in Fig. 5.12B) for each dose condition. **(A)** Peak Time parameter representing the time (in minutes) at which the signal reaches maximum intensity. **(B)** Total AUC. **(C)** Normalized Peak Intensity representing the maximum fluorescence intensity value for each dataset.



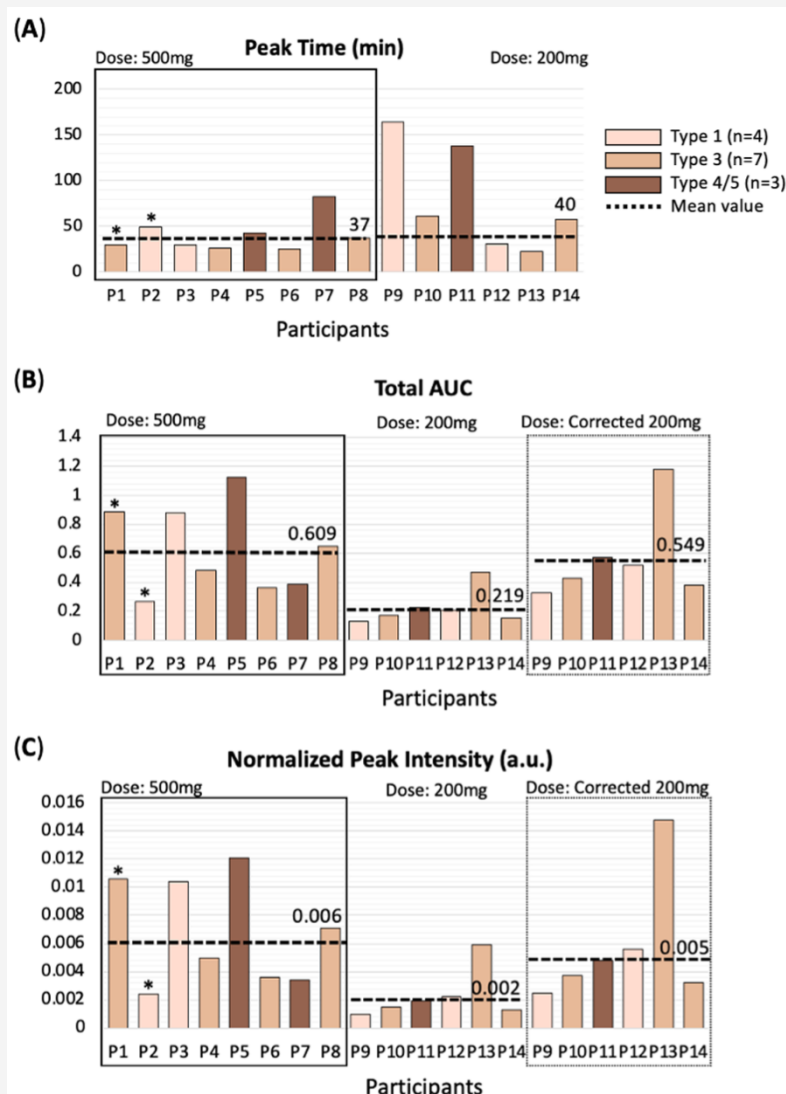
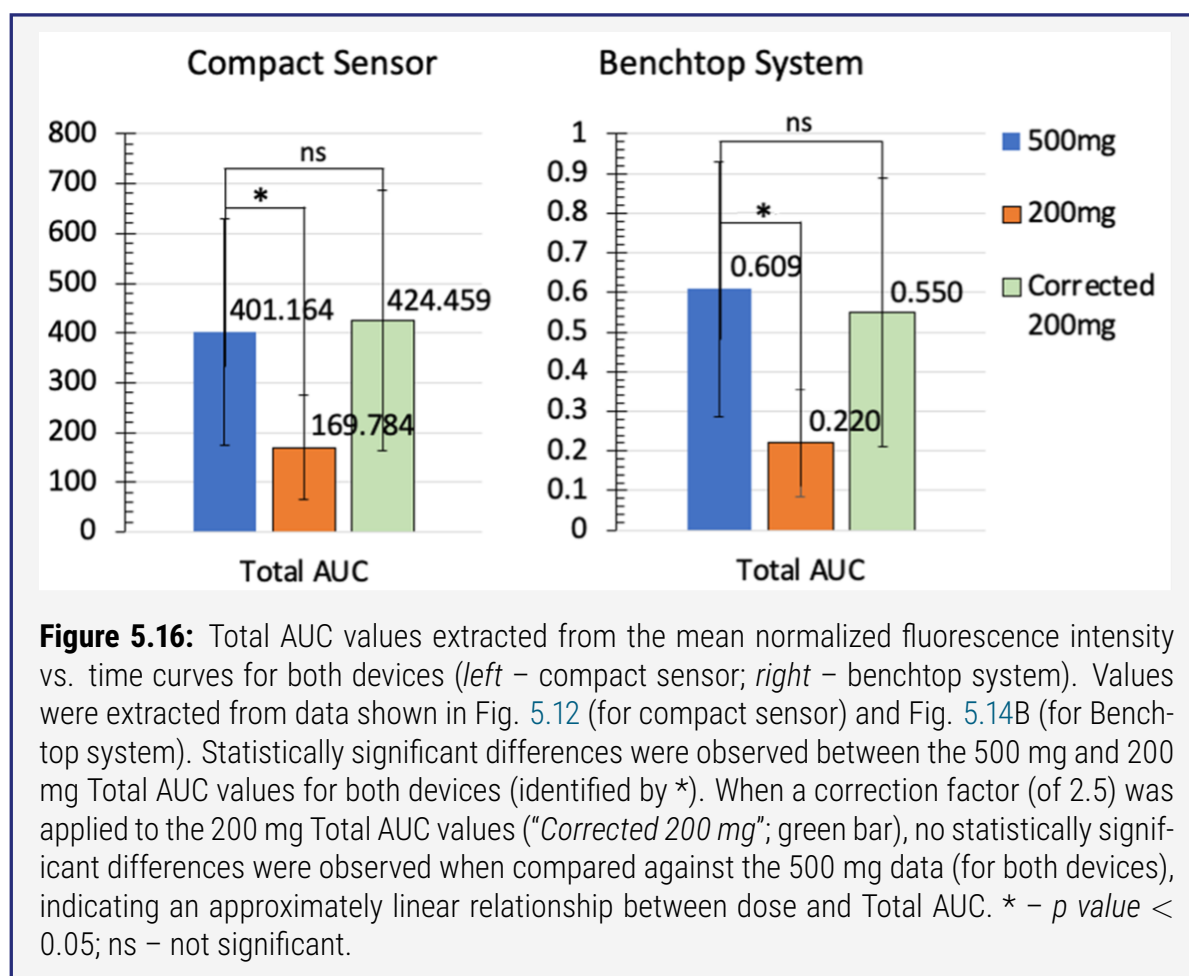


Figure 5.15: Peak Time, Total AUC and Normalized Peak Intensity values calculated from the benchtop system datasets for all participants (P1–14). Values for participants 3–14 were extracted from the full 180 minutes of collected data. Due to early termination of data collection (explained above), parameters for participants 1 and 2 (indicated with an *) were extracted from 135 and 90 minutes of data respectively. On each graph, data is classified according to contrast agent dose (including the “Corrected 200 mg” data, where data collected at 200 mg was multiplied by a correction factor of 2.5 to allow comparison to data collected at 500 mg). Bars are color-coded based on the Fitzpatrick skin tone type for each participant (see legend). The numbers of participants in each Fitzpatrick group are shown in the legend. Dotted lines represent the values from the mean fluorescence vs. time curves (presented in Fig. 5.14) for each dose condition. **(A)** Peak Time parameter representing the time (in minutes) at which the signal reaches maximum intensity. **(B)** Total AUC. **(C)** Normalized Peak Intensity representing the maximum fluorescence intensity value for each dataset.



5.3.2.3 Compact sensor characterization – Impact of skin tone – Stage 3

The final experimental stage (*Stage 3*) aimed to characterize the response of the compact fluorescence sensor when applied to different skin tones. All 14 participants were classified into three groups based on their Fitzpatrick skin tone score (i.e. Fitzpatrick Type 1, Type 3, or Type 4/5). Photographs of the hands and arms of three participants representing Fitzpatrick types 1, 3 and 5 can be seen in Fig. 5.18A.

First, we analysed the first 10 minutes of the data collected in each participant (where fluorescein signals are negligible due to the time delay in fluorescein reaching the blood stream following ingestion). Fig. 5.17A and B show the mean values extracted from the Fluorescence and Backscattered photodiodes (Fig. 5.17A) and the mean normalized intensities (Fig. 5.17B) for each Fitzpatrick group (Type 1, 3 and 4/5). Individual values for each participant are shown in Fig. 5.18. Only minimal (non-significant) differences are observed between Fitzpatrick groups for all parameters (p -value > 0.05 in all cases). However, as expected, the lightest skin type (Type 1) and the darkest skin type (Type 4/5) present the highest and the lowest mean Backscattered Photodiode values (0.451 and 0.329, respectively).

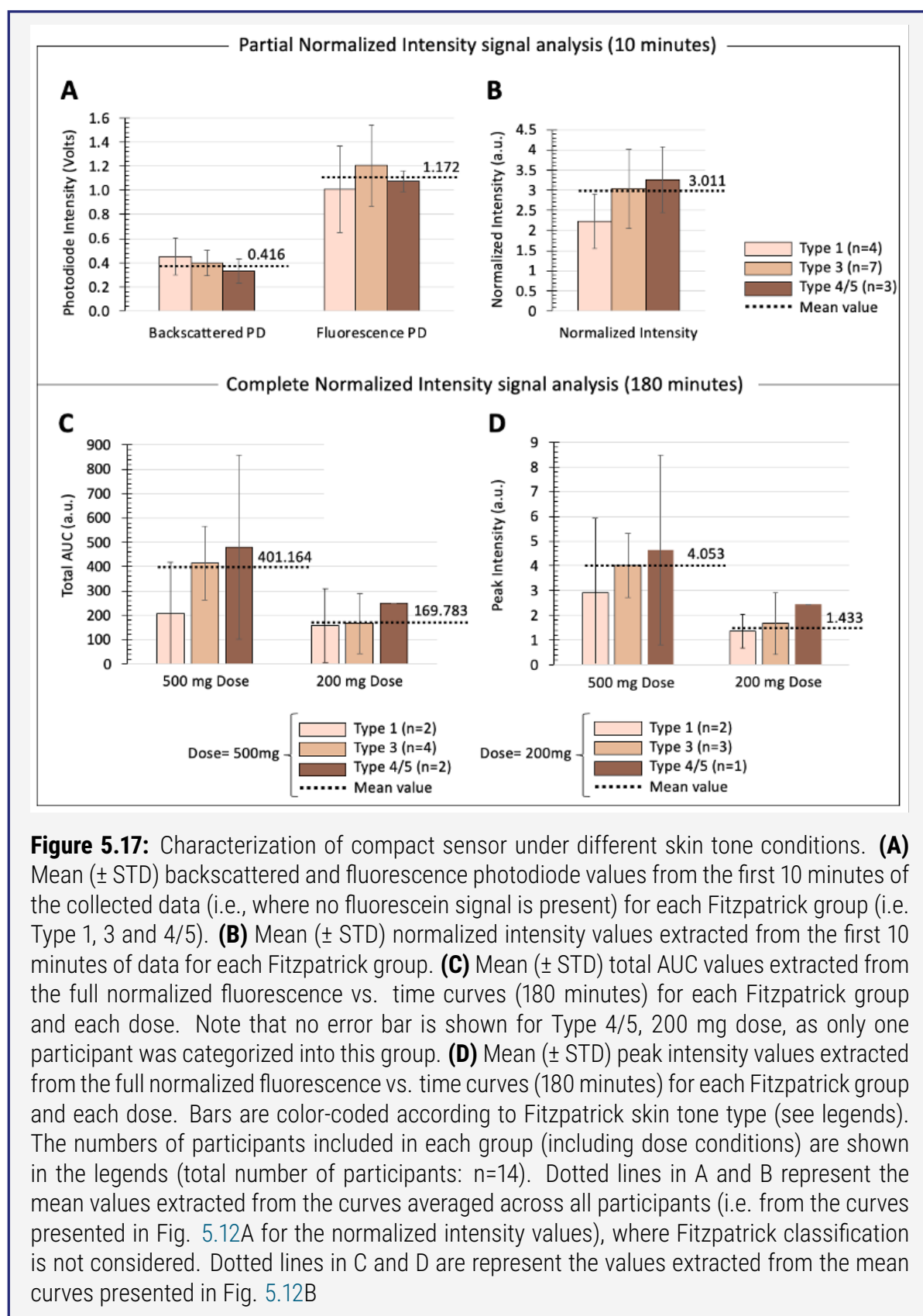


Figure 5.17: Characterization of compact sensor under different skin tone conditions. **(A)** Mean ($\pm$ STD) backscattered and fluorescence photodiode values from the first 10 minutes of the collected data (i.e., where no fluorescein signal is present) for each Fitzpatrick group (i.e. Type 1, 3 and 4/5). **(B)** Mean ($\pm$ STD) normalized intensity values extracted from the first 10 minutes of data for each Fitzpatrick group. **(C)** Mean ($\pm$ STD) total AUC values extracted from the full normalized fluorescence vs. time curves (180 minutes) for each Fitzpatrick group and each dose. Note that no error bar is shown for Type 4/5, 200 mg dose, as only one participant was categorized into this group. **(D)** Mean ($\pm$ STD) peak intensity values extracted from the full normalized fluorescence vs. time curves (180 minutes) for each Fitzpatrick group and each dose. Bars are color-coded according to Fitzpatrick skin tone type (see legends). The numbers of participants included in each group (including dose conditions) are shown in the legends (total number of participants: $n=14$). Dotted lines in A and B represent the mean values extracted from the curves averaged across all participants (i.e. from the curves presented in Fig. 5.12A for the normalized intensity values), where Fitzpatrick classification is not considered. Dotted lines in C and D are represent the values extracted from the mean curves presented in Fig. 5.12B

Second, we analysed the entire datasets (180 minutes) where the overall fluorescence signal is mainly attributed to fluorescein. We extracted the total AUC and the peak intensity values from the

signals presented in Fig. 5.12A and calculated the mean values (and standard deviations) for each Fitzpatrick group. The values can be seen in Fig. 5.17C and D. The individual participant values color-coded by their Fitzpatrick type are presented in Fig. 5.13 and 5.15 for the compact sensor and benchtop system respectively. As above, only minimal, non-significant differences ($p\text{-value} > 0.05$) are observed between Fitzpatrick groups (for both dose conditions).

Taken together, these results suggest that the performance of the compact sensor is not adversely affected by changes in skin tone and indicate that the normalization procedures used (i.e. where the backscattered intensity is used to correct the fluorescence signal level) provide an adequate correction for any changes in fluorescence intensity observed across different tones.

Overall, our results demonstrate strong correlation of data collected with our compact fluorescence sensor with data from an existing benchtop system [90]. This indicates the potential of our compact sensor to transcutaneously assess gut function in a more affordable and portable manner than previously reported. Further characterization of our compact sensor suggested a linear relationship between fluorescein dose and fluorescence signal, exhibiting strong signal-to-noise ratios at fluorescein doses of 200 mg. Importantly, the standard, approved dose of fluorescein used in the clinic is 500 mg (e.g., in fluorescein angiography, where up to 5 ml of fluorescein solution is intravenously injected at a concentration of 100 mg/ml) [186, 234]. Thus, while the use of lower contrast agent doses has the potential to introduce fluctuations in the fluorescence intensity as a result of lower signal-to-noise ratios, our results suggest opportunities to perform transcutaneous spectroscopy using lower fluorescein doses than those typically used in the clinic (i.e. at 200 mg or below). Furthermore, analysis of data with respect to skin tone revealed that variation in skin tone had no discernible effect on the performance of our compact sensor. The ability to detect fluorescence signals under different skin tone conditions and fluorescein doses indicates clear benefits in terms of cost, safety and patient experience, and also suggests the potential to successfully deploy transcutaneous spectroscopy in diverse populations and ethnicities. As such, future steps involved deploying the developed sensor in clinical studies of undernutrition (see Chapter 6 and preliminary results reported in Ref. [229]) to further investigate the potential for non-invasive assessment of gut function.

5.3.3 Miniaturization

5.3.3.1 PCB design and assembly

This final section focuses on advancing the miniaturization of the compact fluorescence sensor presented in the previous sections. This part of the research focused on designing a printed circuit board (PCB) using the Eagle Software (Version 9.6). Emphasis is placed on minimizing the size of the PCB through meticulous configuration of electronics within the board. The board encompasses all the electronic components presented in the final electronic circuit (Fig 5.19B), including the amplifiers and LED driver. The evolution of the PCB design is traced through two versions.

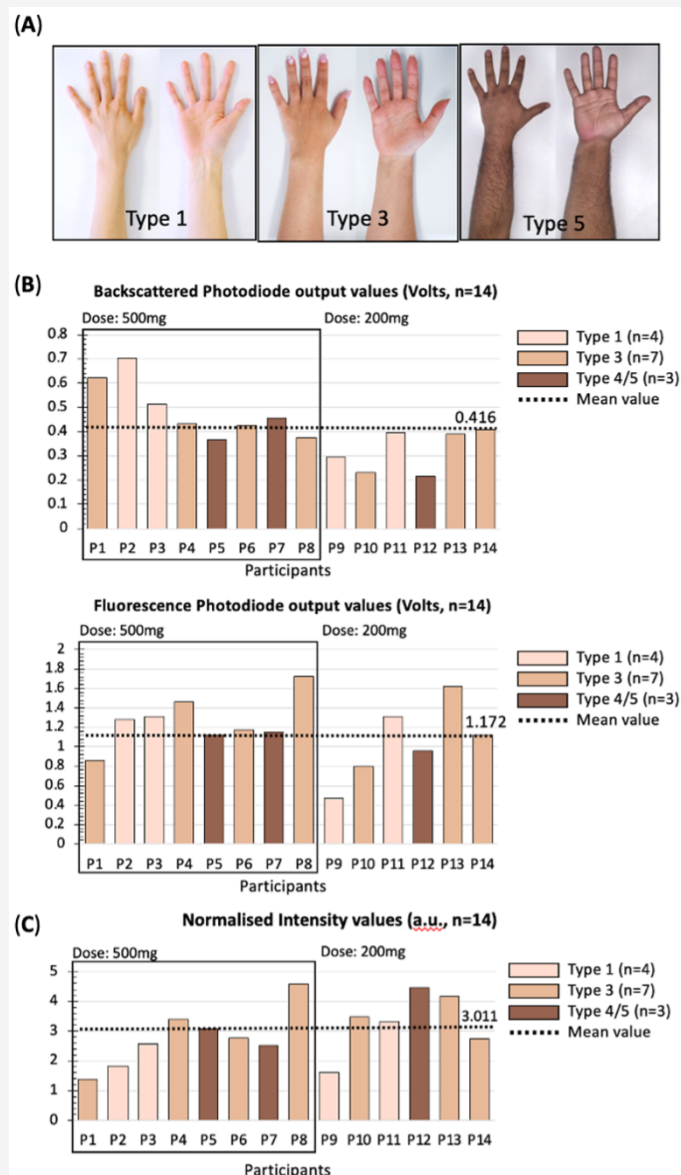


Figure 5.18: Characterization of compact sensor under different skin tone conditions – individual values. **(A)** Photographs of the hands and arms of three participants representing Fitzpatrick types 1, 3 and 5 (left to right respectively). **(B)** Individual backscattered (upper graph) and fluorescence (lower graph) photodiode values averaged over the first 10 minutes of data (i.e., where no fluorescein signal is present). **(C)** Individual normalized intensity values averaged over the first 10 minutes of data (i.e., where no fluorescein signal is present). Bars are color-coded according to Fitzpatrick skin tone type (see legends). The numbers of participants included in each group are shown in the legends (total number of participants: n=14). Fluorescein doses used for each participant are indicated on the graphs. Dotted lines represent the mean values extracted from the values averaged across all participants (i.e., with Fitzpatrick classification not considered).

Fig. 5.19B and D shows both PCB designs, together with the sensor used for the clinical studies described in previous versions (5.19A). Initially, the first version incorporated all the electronic components, including both Photodiodes, but only the connections for the high-power LED were

included, due to its size. Fig. 5.19B shows the first PCB design with a description of all the main parts and a size comparison between the PCB, the LED and a SMA fibre connector (5.19C) showing at their true size. A GUARD was incorporated in the design to create a protective barrier around the photodiodes' connections due to the small output current ($0.223 \mu\text{A}$). The primary function of a GUARD is to shield certain parts of the circuit preventing interferences or unintended coupling with other parts of the circuit during hand-soldering, as in this case. In addition, GUARDS help in minimizing electromagnetic interferences between adjacent components, reducing the risk of crosstalk and signal degradation. However, this first PCB design lacked the GUARD around the Photodiodes and overlooked the specific dimensions of the electronic components, such as resistances and capacitances. In addition, the attachment of the SMA connectors to the Photodiodes also posed a physical obstacle due to their size differences (Fig. 5.19C). After recognizing these practical challenges, the second PCB design only included pins to connect the photodiodes and the LED, which were fully shield with the GUARD. This configuration offers the possibility to isolate the optical components (Photodiodes, optical filters and LED) to ensure an improved connection with the custom fibre probe through the use of SMA connectors. Fig 5.19C shows the second PCB design. As before, the main parts of the circuit are labelled in the PCB.

This final PCB design is now implemented in a more compact, wearable version of the sensor presented in this thesis, developed in collaboration with Dr. Nilanjan Mandal. Fig 5.19E shows the current version of the fluorescein sensor that incorporates a touchable screen for raw data visualization (using the WIO terminal), an 850 mmhA battery and a SD card module where raw data is stored. No pre-processing step is implemented in this version of the sensor. However, ongoing and further work focuses on improving data collection and storage, battery consumption and advanced miniaturisation.

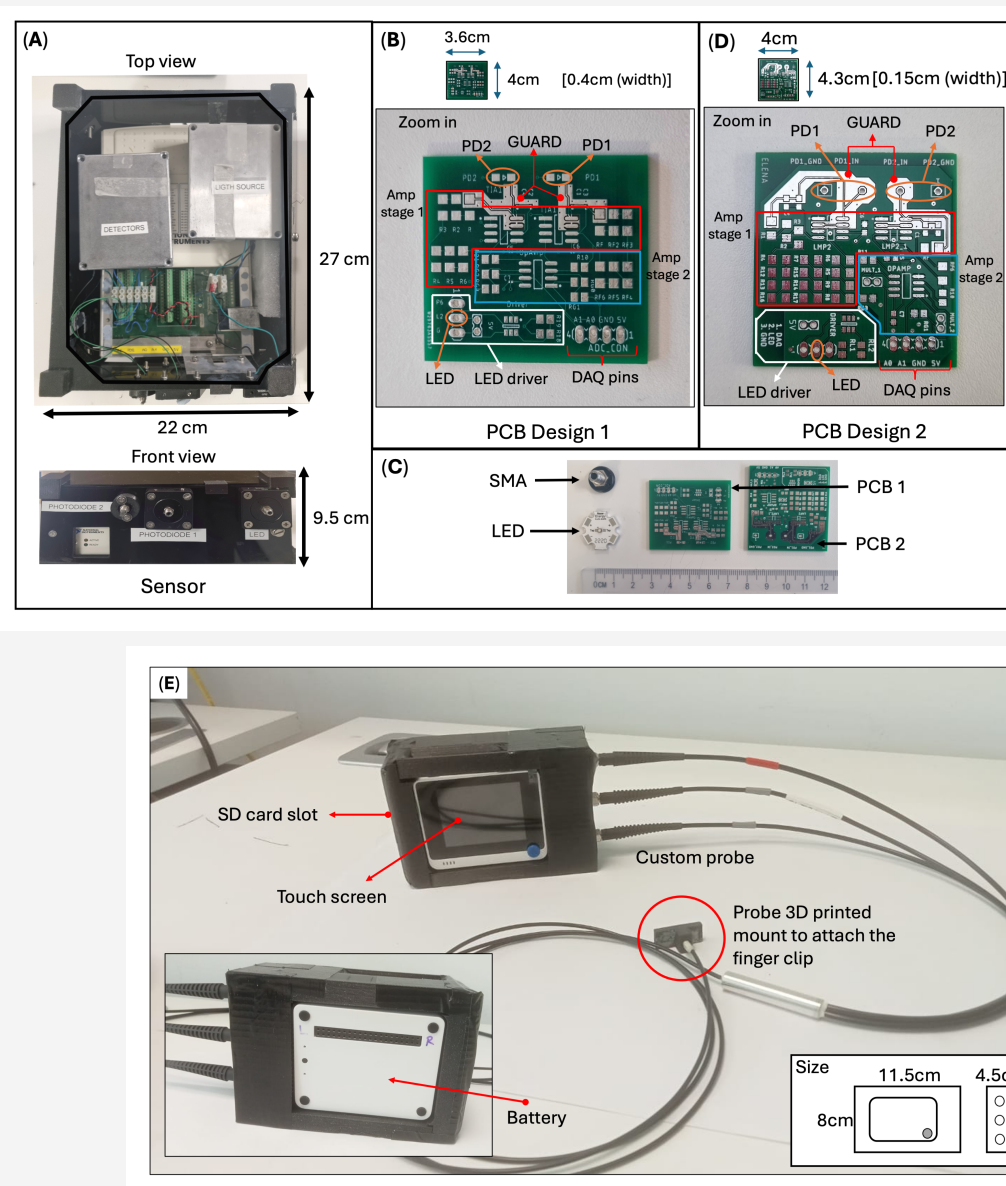


Figure 5.19: Miniaturisation of the compact optical sensor. **(A)** Initial configuration of the compact sensor presented in the previous sections. Top and front view are shown. The top view also depicts the internal electronic configuration of the sensor, showcasing the differences in size between the electronics (integrated inside the metallic boxes) and the NI DAQ used to control and collect data. **(B)** First PCB design. Internal parts, including the amplification stages (denoted as “Amp stage”) and the LED driver, are identified. **(C)** Size comparison between the PCB designs and the LED and SMA connectors. **(D)** Final PCB design. As before, internal parts, including the amplification stages and the LED driver, are identified. **(E)** Current version of the miniaturisation developed in collaboration with Dr. Nilanjan Mandal. It integrates the final PCB design shown in (D). A battery source, as well as a touchable screen (WIO terminal) and an SD card port are included. As in the compact sensor shown in (A), the custom fibre probe is attached to the photodiodes and LED through SMA connectors. The distal part of the probe is then attached to the participant using a 3D printed finger clip (not present in the image).

5.4 Conclusion

This chapter has described the development of a compact optical sensor that provides measurements of fluorescence intensity during the absorption and elimination of fluorescein in the bloodstream after oral ingestion. The system, fully housed in a laser-cut plastic structure, uses a [LED](#) for excitation of fluorescein with an emission wavelength of 465 nm. It has been designed for ease of portability and to make clinical use safe and practical. The in-house written Labview software allows for full control and use of the sensor by a single user. The sensor has been, initially, validated *in vitro* to later be deployed in St Mary's Hospital for further *in vivo* validation in healthy controls. To date, the sensor has been involved in 25 healthy *in vivo* measurements of fluorescein intestinal permeation (14 of those have been presented in this chapter). The preliminary *in vivo* results presented here exhibited agreement with a benchtop spectrometer (previously described in Ref. [90]) while providing clear advantages in terms of portability and cost (estimated costs of the benchtop system and the compact sensor were 10k and 2k). Further *in vivo* analysis demonstrated the performance of our compact sensor under different contrast agent doses, suggesting a linear relationship between the ingested dose and the observed fluorescence intensity. In addition, we characterized the sensor's response when applied to different skin tones, with results indicating minimal impact of skin tone on sensor performance. The observations made during the *in vivo* validation demonstrate the potential of our compact, transcutaneous fluorescence sensor to provide non-invasive assessment of gut function and suggest that the use of a miniaturized version of the sensor could particularly be useful for larger-scale clinical studies. Following this consideration, further work was focused on sensor miniaturisation, proving to reduce the size of the sensor considerably and potentially facilitating deployability. Future work in this field is directed towards the development of a fully functional miniaturized version of the device. Indeed, work towards the investigation of fluorescence intensity profiles in pathologic stages, such as undernutrition, had already been started (See Chapter 6). More immediate future work will involve the collection of a larger dataset to continue validating the device and to assess the relationship between fluorescein intensity profiles and gut permeability, including investigation in other body areas and under different motion conditions.

Chapter 6

Clinical validation of a compact fluorescence sensor: monitoring gut permeability in healthy and undernourished populations

The previous chapter presented the development of a compact fluorescence sensor, and the results demonstrated the potential of the system to allow for monitoring the permeation of a fluorescence contrast (fluorescein) from the gut to the bloodstream. The previous chapter also analysed the impact of motion and optical artifacts can have in the fluorescence intensity signals. This chapter now concentrates on further *in vivo* validation of the compact sensor, focused on two experimental groups with expected to exhibit different gut permeabilities. Further experimental work includes the analysis and correlation of current clinical standard techniques, such as permeability tests and endoscopic biopsies, with our sensor. The results will provide a better understanding of the capabilities of the compact sensor to monitor and diagnose changes in gut permeability.

6.1 Introduction

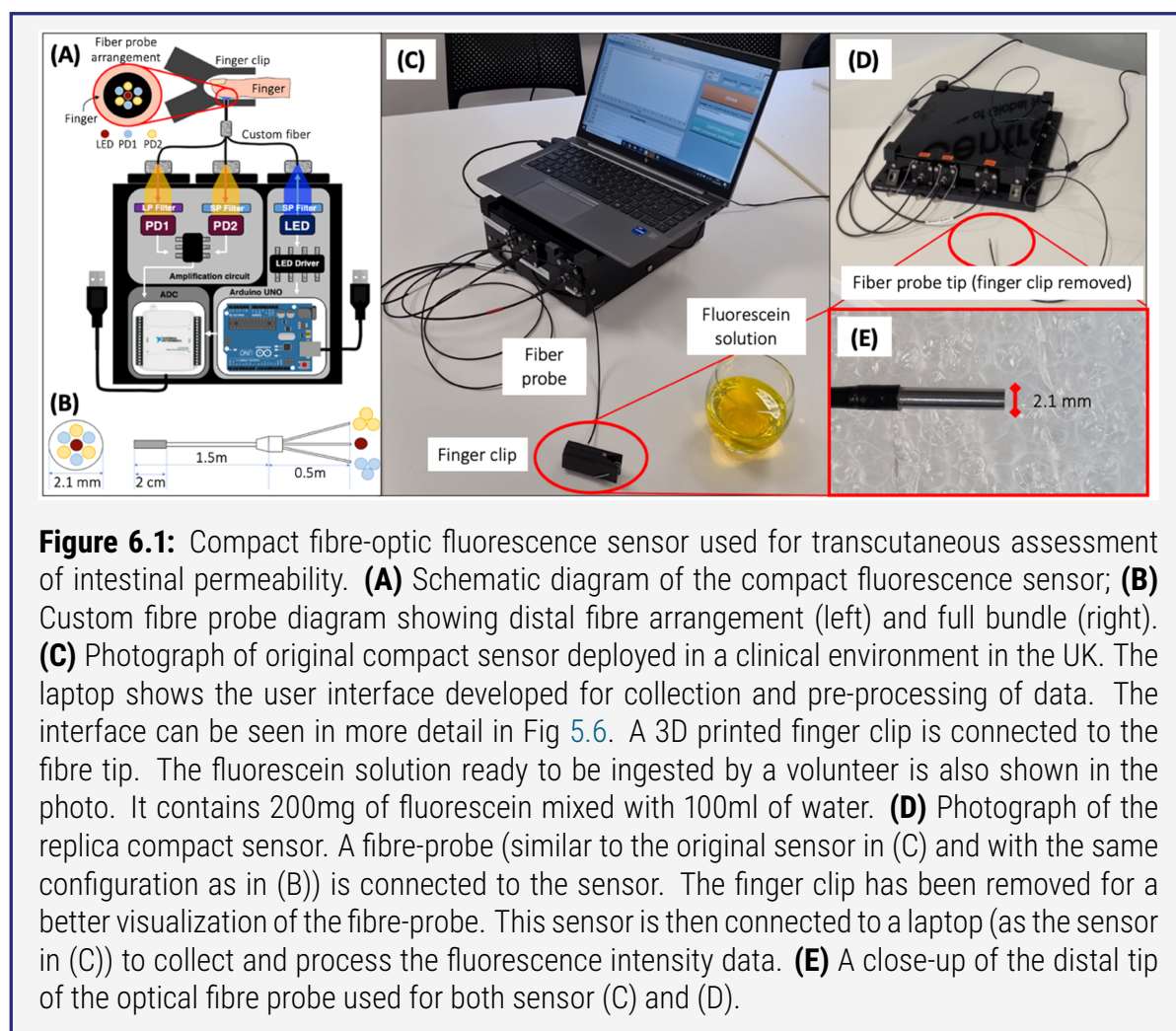
Undernutrition stands as a global health concern with multifaceted implications that affect critically regions in sub-Saharan Africa and South Asia [74, 75]. The most current and specific data can be found in reports from organizations such as the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) [61, 76]. As stated by the WHO, undernutrition is a condition marked by an inadequate intake of essential nutrients crucial for maintaining health and well-being [62–64]. An inadequate absorption of nutrients is often associated with a compromised intestinal barrier, which can result in an increased gut permeability and the passage of bacteria, toxins and undigested food particles from the gut into the bloodstream, triggering an inflammatory response [235, 236].

As explained previously, current clinical procedures to diagnose and monitor abnormal gut permeability include permeability assays and endoscopic biopsies. Briefly, quantitative sugar permeability assays, such as Lactulose:Mannitol test, evaluates the integrity of the intestinal barrier by measuring the recovered lactulose and mannitol or rhamnose concentrations in urine after oral ingestion of the sugars [125–127, 131, 135, 237, 238]. Although previous studies showed the potential of both L:M and L:R tests for non-invasive assessment of GI permeability, their applicability in clinical research is currently limited due to lack of standardizations in their methodologies [130, 142]. Alternatively, endoscopic biopsy and histopathology can provide a direct examination of the intestinal lining. However, they are highly invasive, difficult to perform in infants and carry inherent risks [112, 239]. In addition, laboratory-based approaches such as these lead to delays between administering the tests and reporting results. Recent clinical and preclinical studies demonstrated the potential of transcutaneous fluorescence spectroscopy for non-invasive monitoring gut permeability and other GI functions [90, 145–148, 209]. In previous chapters, we have seen that using laser-based spectrometers, which are both large and expensive, can introduce additional challenges in certain patient groups and environments, thereby precluding widespread deployment [90, 145, 146, 209]. In Chapter 5, we demonstrated the potential of a compact fluorescence spectroscopy sensor for non-invasively monitoring gut permeability, therefore, facilitating the clinical uptake of transcutaneous spectroscopy on a larger scale [229, 230]. To further demonstrate the clinical potential of the compact fluorescence sensor for non-invasive assessment of gut permeability, this chapter reports results from a clinical study on healthy and undernourished volunteers. As reported in the introduction, undernutrition stands as a global health concern. The analysis of fluorescence spectroscopy data from both healthy subjects and those experiencing undernutrition will further provide information about this condition and the potential of our compact sensor for non-invasive assessment of gut function in undernutrition population. This chapter also includes a comparison with current clinical approaches (i.e. L:M and L:R permeability assay and duodenal biopsies), offering valuable insights into the limitations of these techniques and their correlation with our compact sensor for the non-invasive assessment of gut permeability.

6.2 Methods

6.2.1 Compact transcutaneous fluorescence sensor

Following oral ingestion of fluorescein, a compact fibre-optic fluorescence sensor (previously presented in Chapter 5) was used to detect fluorescence signals at the fingertip. The optical setup of our compact sensor, as illustrated in Fig. 6.1A, consists of a 465 nm LED for excitation and two photodiodes (Fluorescence Photodiode and Backscatter Photodiode) with peak sensitivity at 620 nm. The optical path is controlled using flexible reflective optical filters in front of the LED and photodiodes. The Backscatter Photodiode is employed to calculate the normalized fluorescence signal to correct for variations due to factors such as probe orientation, fluctuations in excitation power, and variations in skin colour and thickness. Both Photodiodes and the LED are connected to a custom trifurcated fibre-optic probe, enabling the interrogation of fluorescence signals at the skin. The probe includes 7 multimode silica optical fibres, arranged to maintain a consistent interrogation volume for both photodiodes (as illustrated in Fig. 6.1B).



To secure the distal tip of the custom fibre probe to the participant's forefinger during measurements, a 3D-printed finger clip was designed with a loaded spring mechanism, ensuring constant contact pressure and minimizing motion artifacts (Fig. 6.1C). The system is driven and controlled by a custom-written LabVIEW interface, automating data collection and pre-processing. A replica of the compact sensor depicted in Fig. 6.1C was developed in order to investigate healthy and undernutrition subjects simultaneously (i.e., to allow experiments to be performed in both Zambia and the UK). The original device (from now referred to as Sensor 1), inclusive of the sensor, fibre probe, finger clip, and the LabVIEW control software running on a laptop, is depicted in Fig. 6.1C. The replica sensor (from now referred to as Sensor 2) and its fibre probe are shown in Fig. 6.1D. Fig. 6.1E shows a close-up of the distal tip of the optical fibre probe used in the replica sensor. A similar fibre probe was used on the original sensor (shown in Fig. 6.1B). The sensors record both the fluorescence signal and the backscattered signal which is then processed automatically in the interface. The processing involves a normalization – which is described in detail in Chapter 5 and Ref. [90] – that allows for calculation of a fluorescence vs. time curve, which can be analysed to extract information regarding gut permeability and other elements of GI function.

For the clinical experiments reported here, both sensors were programmed to record the fluorescence and backscattered signals once every 20 seconds for a total of 3 hours. This allowed for calculation of fluorescence vs. time curves with sufficient temporal resolution to observe the dynamics of the fluorescein signal (e.g. the permeation from the gut into the blood stream and the elimination of the dye from the blood). In turn, this permitted calculation of a set of parameters designed to provide readouts of gut permeability and previously reported in Chapter 4.

6.2.2 Experimental procedures

6.2.2.1 Calibration

The compact sensors depicted in Fig. 6.1C and D share a common electronic configuration, as illustrated in Fig. 6.1A. While they exhibit hardware and electronic similarities, it is important to note that they are not entirely identical. Both compact sensors are assembled using commercially available off-the-shelf components, but the availability of certain components (such as the data acquisition system (DAQ)) posed a challenge. For this reason, Sensor 1 was equipped with a NI USB-6289 DAQ, whereas Sensor 2 incorporated a NI USB-6212 DAQ. Both have a resolution of 16 bits, which determines the accuracy of the sensor readings, but NI USB-6289 supports higher maximum sampling rate (625 kS/s vs 400 kS/s) that might capture more finite signal changes. These differences between the DAQs could potentially impact the comparison of the resulting signal from the sensors. In addition to these differences, for Sensor 2, the electronic gain on the second amplification stage of the Fluorescence Photodiode was slightly reduced (from 8.977 dB to 6.232 dB) to mitigate potential issues during the deployment of the sensor in remote locations, where technical oversight is limited (see Section 6.2.2.2 for details). The modification of this gain made the total

electronic gain of the Sensor 2 (107.993 dB) different from Sensor 1 (110.737 dB). Fig. 6.2 shows the comparison between Sensor 1 and Sensor 2 electronic circuits (modifications on Sensor 2 are highlighted in red). This adjustment serves to prevent the saturation of the fluorescence intensity signal and, by extension, the risk of losing critical data concerning variations in fluorescein permeation. It is worth noting that this modification may have an impact on the resolution of the sensor, particularly the Signal-to-Noise Ratio, in capturing physiological fluctuations in the fluorescence intensity signal. The resulting Signal-to-Noise ratio is 13.001 dB, in comparison with the 13.384 dB from Sensor 1.

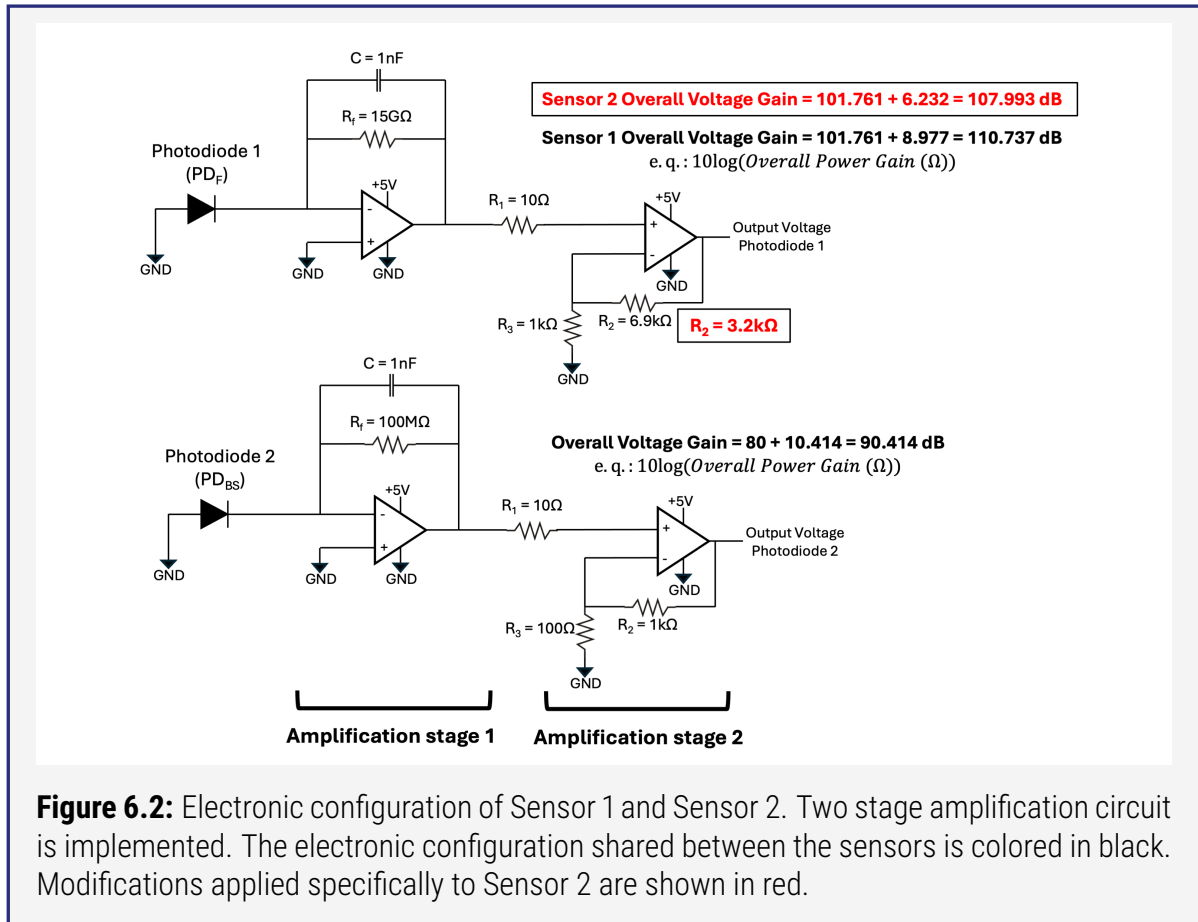
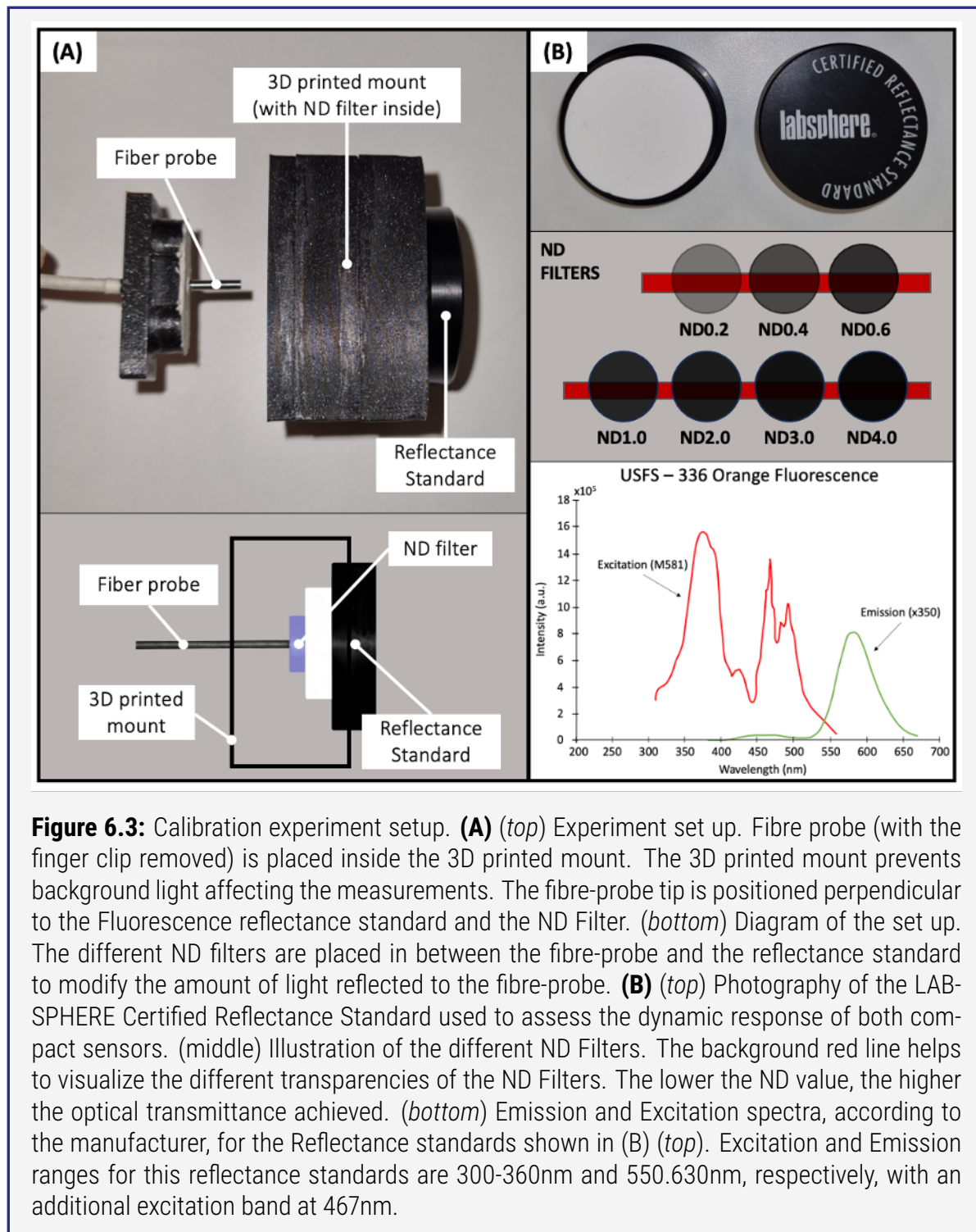


Figure 6.2: Electronic configuration of Sensor 1 and Sensor 2. Two stage amplification circuit is implemented. The electronic configuration shared between the sensors is colored in black. Modifications applied specifically to Sensor 2 are shown in red.

The rapid sampling rate incorporated in both sensors (100 samples per second and one normalized data point recorded every 20 seconds) can help to mitigate noise levels through oversampling the data, thereby improving resolution. However, the modifications in Sensor 2 still suggest an impact on the normalized fluorescence intensity signal and direct comparison between both sensors is deemed impractical and unrealistic. Therefore, a calibration step is introduced prior to conducting clinical trial measurements. This step aims to derive a Calibration factor necessary to enable realistic comparison of datasets collected from both compact sensors, specifically the analysis of fluorescence intensity values among distinct subject groups (i.e., healthy and undernourished subjects). The calibration setup is visually represented in Fig. 6.3A. This setup incorporates a Fluorescence Reflectance Standard (LABSPHERE, Certified Reflectance Standard, 300-360 nm excitation

and 560-630 nm emission, Fig 6.3B), alongside a range of Neutral-Density (ND) Filters (ranging from ND = 0.2 to 4, Fig. 6.3B). All these components are used to assess the dynamic response of both compact sensors to a series of fixed fluorescence intensities. The values ascertained during the calibration process are subsequently employed to calculate a Calibration Factor.



To validate the calculation, this Calibration factor is evaluated on a series of fluorescence samples (previously described in Chapter 5). As explained in Chapter 5, these solutions were designed to approximate the fluorescence intensity observed in vivo (based on data collected using a benchtop system) and they are not intended to act as realistic optical phantoms. The fluorescein concentrations used were 0 mg/ml for the baseline (water with no fluorescein), 0.0044 mg/ml, 0.0056 mg/ml, 0.00083 mg/ml and 0.0017 mg/ml. Fluorescence signals from each solution were collected using both compact sensors simultaneously.

6.2.2.2 Clinical trial measurements

Clinical trial experiments were focused on analysing the potential of our compact sensor on assessing gut permeability differences between healthy and potential undernutrition participants. To facilitate this investigation, two similar compact sensors (presented in Section 6.2.1) were used. Sensor 1 was deployed in St. Mary's Hospital (London, UK) between September 2022 and August 2023 to collect fluorescence intensity data from 17 Healthy participants (Group 1). Healthy volunteers were screened before participation to confirm that they did not have a diagnosis of any known conditions that altered gut permeability. The fluorescence intensity measurements were performed according to a local clinical study protocol [220] (details of ethical approval: UK Health Research Authority IRAS Project ID – 242462; Research Ethics Committee reference – 18/LO/0714/AM04). Sensor 2 was deployed in St. Augustine Clinic (Lusaka, Zambia) and experiments were performed in 80 volunteers, following Ethical approval from the University of Zambia Biomedical Research Ethics Committee, 2291-2021, dated 20th December 2021. Out of the 80 participants recruited in Lusaka, Zambia, fluorescence data was collected in 34 participants. Participants from group 2 were also screened before participation. In this case, the presence of any pathology associated with gut dysfunction was evaluated. All experiments were performed in accordance with Good Clinical Practice (GCP) guidelines and the World Medical Association's Declaration of Helsinki. All volunteers gave informed consent prior to the experiments, all participants were aged 18 and above and had no previous reactions to fluorescein. Participants who were pregnant, were breast feeding, or had taken antibiotics for the last 4 weeks were excluded. The participant demographics for each group are tabulated in Table 6.1 and 6.2.

For the fluorescence spectroscopy experiments, both sensors were deployed in their respective clinical set ups: Sensor 1 was located in St Mary's hospital (London, UK) and Sensor 2 was deployed in St Augustine clinic (Lusaka, Zambia). Fig. 6.4 shows Sensor 2 deployed in St Augustine Clinic (Zambia) for the Undernutrition study. For all volunteers (healthy and undernourished), fluorescence fibre probes (from Sensor 1 and Sensor 2, respectively) were first attached to the participant's first finger. Fluorescence measurements were then started at the same time that the participant was asked to begin drinking a fluorescein solution containing 200 mg fluorescein dissolved in 100 ml water.

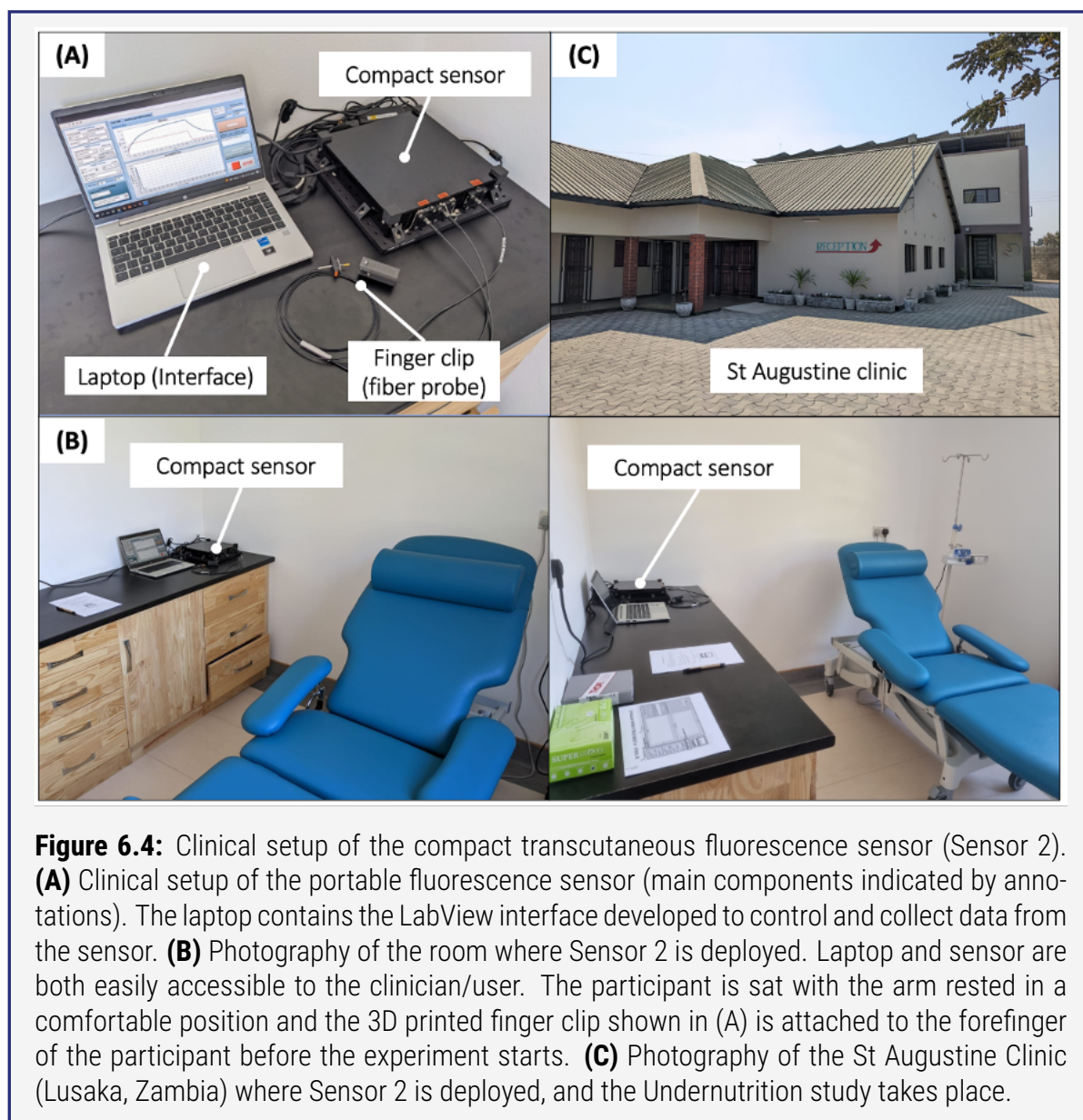


Figure 6.4: Clinical setup of the compact transcutaneous fluorescence sensor (Sensor 2). **(A)** Clinical setup of the portable fluorescence sensor (main components indicated by annotations). The laptop contains the LabView interface developed to control and collect data from the sensor. **(B)** Photography of the room where Sensor 2 is deployed. Laptop and sensor are both easily accessible to the clinician/user. The participant is sat with the arm rested in a comfortable position and the 3D printed finger clip shown in (A) is attached to the forefinger of the participant before the experiment starts. **(C)** Photography of the St Augustine Clinic (Lusaka, Zambia) where Sensor 2 is deployed, and the Undernutrition study takes place.

A dose of 200 mg was chosen to reduce any potential side-effects (as it is below the clinical approved dose (500mg) of fluorescein for other medical procedures [186]). In addition, previous work presented demonstrated good signal levels for orally ingested fluorescein of 200mg (see Chapter 5 and Ref. [229, 230]). After ingestion of the fluorescein solution, fluorescence signals were recorded for a total of 180 minutes, with normalized fluorescence intensity values calculated and reported every 20 s. In both experimental set ups (Group 1 and 2), all volunteers were seated for the duration of the experiments and all volunteers fasted overnight prior to the experiment.

Table 6.1: Demographics of the study participants in Group 1 Fluorescein dose – 200 mg (in 100 ml water). Lower region of table presents the percentages and numbers of participants in each Fitzpatrick skin tone. BMI scores are also included.

ID	Age	Sex	Fitzpatrick class	BMI
UK1	24	F	1	-
UK2	27	M	2	24.8
UK3	23	F	1	-
UK4	37	M	5	30.8
UK5	23	F	3	18.5
UK6	23	M	3	23.5
UK7	32	M	3	21.9
UK8	26	M	5	24.1
UK9	35	M	6	24.7
UK10	26	M	5	-
UK11	25	M	5	22.1
UK12	21	M	5	19.8
UK13	38	F	2	19.7
UK14	62	M	3	27.4
UK15	39	M	3	25.2
UK16	31	M	2	-
UK17	36	M	3	28.1
Male / Female			23.529%/76.471%	
Fitzpatrick class 1 and 2			29.411% (5)	
Fitzpatrick class 3 and 4			35.294% (6)	
Fitzpatrick class 5 and 6			35.294% (6)	

Table 6.2: Demographics of the study participants in Group 2 Fluorescein dose – 200 mg (in 100 ml water). Lower region of table presents the percentages and numbers of participants in each category. BMI scores are also included.

ID	Age	Sex	BMI	ID	Age	Sex	BMI
Z1	32	F	24.20	Z18	31	F	29.2
Z2	23	M	20.5	Z19	19	M	20.7
Z3	43	F	25.8	Z20	45	F	26.7
Z4	28	F	20	Z21	35	F	31.3
Z5	31	M	25.6	Z22	22	M	19.1
Z6	32	F	20.7	Z23	21	F	25.3
Z7	52	M	21.5	Z24	30	F	26.9
Z8	60	M	26.3	Z25	20	M	21.1
Z9	29	M	25.2	Z26	26	F	32.4
Z10	28	F	28.4	Z27	36	F	21.8
Z11	22	M	24.6	Z28	22	M	20.8
Z12	19	F	28.8	Z29	21	M	19.9
Z13	28	F	26.3	Z30	25	M	23.1
Z14	34	F	20.6	Z31	46	F	25.2
Z15	46	M	21.9	Z32	36	F	39.7
Z16	52	M	19.8	Z33	30	F	38.6
Z17	29	F	23.2	Z34	23	M	20.2
Male / Female				55.882%/44.117%			
Fitzpatrick class 1 and 2				0			
Fitzpatrick class 3 and 4				0			
Fitzpatrick class 5 and 6				100% (34)			

6.2.2.3 Permeability assays - sugar molecules

Quantitative sugar permeability assays such as Lactulose:Mannitol and Lactulose:Rhamnose tests have the potential for non-invasive evaluate intestinal permeability [125–127, 131, 135, 237, 238]. However, their clinical application is limited due to their practicality and lack of standardization between studies [130, 142]. In this Chapter, we also aimed to compare the performance of current sugar permeability assays (L:M and L:R) and our fibre-probe compact sensors in both healthy and undernourished groups. By doing so, we address certain limitations associated with the existing standard permeability tests (L:M and L:R) and, further, demonstrate the potential of our sensor for non-invasively assess gut permeability.

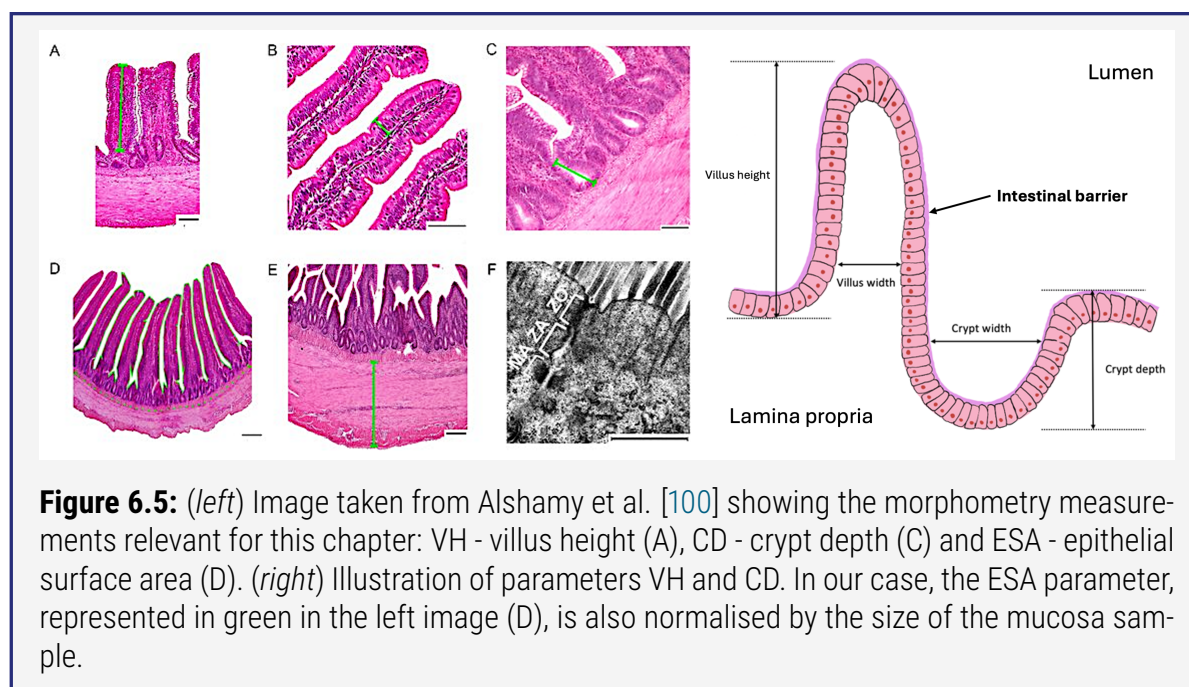
For the permeability assays, all participants were requested to empty their bladders into a collection container (baseline levels) before ingesting the respective test sugar solutions. Due to variations in the protocols between healthy and undernourished groups, L:M test and L:R were performed separately on each group, respectively. Volunteers from group 1 undertook the L:M permeability assay no less than a week after the transcutaneous fluorescence experiment. On the contrary, volunteers from group 2 undertook the L:R permeability assay at the same time as the transcutaneous

fluorescence experiment.

The Lactulose:Mannitol (L:M) test was performed in a cohort of 17 healthy participants, with prior confirmation of any known conditions that altered gut permeability. A total of 15 urine samples (12 male and 3 female) were collected at 6 hours after L:M solution administration. The Lactulose:Rhamnose (L:R) test was performed in the undernourished group. From the 34 participants whose fluorescence signals were available, 14 urine samples (6 males and 8 females) were collected at 3 hours after L:R solution administration. After collection of urine samples in both healthy and undernourished volunteers, total urine volume was measured and recorded. Samples were then stored in 1ml aliquots at a -80°C freezer until Lactulose:Mannitol and Lactulose:Rhamnose concentrations could be determined independently. The samples were analysed in the MRC-NIHR BRC National Phenome Centre (at Hammersmith hospital – Imperial College).

6.2.2.4 Endoscopic biopsies

In conjunction with the assessment of fluorescence intensity signals and L:R samples, endoscopic biopsies were collected from participants comprising Group 2 on the same day of the fluorescence study. Following the collection of the L:R urine samples, the participants were translated to an endoscopic room where biopsies were collected. These biopsies provide an anatomical representation of a portion of the gastrointestinal wall at the duodenum, providing a comprehensive understanding of the gastrointestinal condition of the participants.



The biopsies were processed and analysed at St Augustine clinic (Lusaka, Zambia). The research team conducted a detailed examination, extracting crucial parameters such as villus height (VH), crypt depth (CD), crypt-to-villus ratio (CVR) and epithelial surface area (ESA). Fig. 6.5 shows an

illustration of the parameters extracted from the biopsies. [ESA](#) measurements were normalised by the total length of the tissues sample (i.e. mucosa muscularis) and multiplied by 100 to have a measurement per 100 μm . These parameters, vital for elucidating the histological characteristics of the gastrointestinal mucosa, were then provided by the research group in Lusaka to compare with the additional parameters collected from the participants in Group 2 (i.e., fluorescence intensity signals and [L:R](#) urine samples).

6.2.3 Statistical analysis

Following the calibration of the sensors, statistical tests were first performed to analyse the correlation between the signals from the sensors and the permeability tests. This included the comparison between the compact sensors' fluorescence signals and their respective [L:M](#) or [L:R](#) samples, as the standard approaches to assess intestinal permeability [[125–127](#), [131](#), [135](#), [237](#), [238](#)]. In addition, correlation between the morphometry data and the fluorescence intensity signals from group 2 was also calculated. Further analysis then focused on assessing the performance of the compact sensors to distinguish various gut conditions, which included the comparison between the dataset from group 1 (healthy) and group 2 (undernourished).

Normalised fluorescence intensity signals, as well as parameters extracted from them - including the total area under the curve (Total [AUC](#)), peak intensity, Peak Time, Peak [AUC](#), Peak [AUC](#)/Slope and Peak [AUC](#)*Peak Time – were used for the analysis. In the case of permeability test, percentages of Lactulose, Mannitol and Rhamnose recovered in the urine (i.e. %L, %M and %R), as well as the Lactulose:Mannitol ratios and Lactulose:Rhamnose ratios were used. Morphometry parameters extracted from the biopsies – Villus Height ([VH](#)), Crypt Depth ([CD](#)) and Epithelial Surface Area ([ESA](#)) – were also compared against the fluorescence signals and the Lactulose:Rhamnose test.

For the analysis, Pearson's correlation coefficients (r) were used to measure the linear correlations between the compact fluorescence sensor values and permeability markers, including the morphometry parameters. P -values < 0.05 were used to infer statistically significant correlations. Pearson's correlation coefficients (r) were calculated using the "*corrcoef*" MATLAB function. Additionally, unpaired Student t-tests were used to analyse differences between the studied groups (healthy and undernourished) using the data collected with each compact sensor (Sensor 1 and Sensor 2), as well as the permeability markers ([L:M](#), [L:R](#) values and morphometry parameters).

6.3 Results

6.3.1 Compact fiber-optic sensor calibration

As explained on Section 6.2.2.1, both developed compact sensors (Sensor 1 and Sensor 2) have the same electronic configuration. However, some modifications (explained in Section 6.2.2.1) were performed in Sensor 2 as per requirement on the experimental protocol and limitations on the components available during development. In order to account for these differences and to be able to directly compare data obtained from both sensors, a calibration steps was incorporated. A standard reflectance and different ND filters were used in order to analyse the dynamic response of the fluorescence and backscattered photodiodes to different light profiles. The purpose of the standard ND filter, in this case, is to reduce the amount of light entering the detector, so we can characterize the dynamic response of the compact sensors to different fluorescence intensities. The amount of fluorescence and backscattered light transmitted through the ND filter and to the detector (photodiodes) is determined by the optical density (d) of the ND filter. The Fractional Optical Transmittance (FOT) of each filter is calculated following equation (6.1):

$$FOT = \frac{I}{I_0} = 10^{-d} \quad (6.1)$$

, where I is the intensity after the ND filter, I_0 is the Intensity before the ND filter and d is the optical density of the ND filter. The optical density, d , and transmittance of each ND filter is shown in Table 6.3.

Table 6.3: Optical density and Fractional optical transmittance (FOT) of each ND Filter used to calculate the Calibration Factor.

Filter	Optical Density (OD)	Fractional Optical Transmittance (FOT)
ND 0	0	1 [100 %]
ND 0.2	0.2	0.630 [63.00 %]
ND 0.4	0.4	0.398 [39.80 %]
ND 0.6	0.6	0.251 [25.10 %]
ND 0.8	0.8	0.158 [15.80 %]
ND 1.0	1.0	0.100 [10.00 %]
ND 2.0	2.0	0.010 [1.00 %]
ND 3.0	3.0	0.001 [0.10 %]
ND 4.0	4.0	0.0001 [0.01 %]

In order to account for noise fluctuations, multiple repeats ($n=10$), on three different days (total of 30 repeats), were collected. Fig 6.6A shows the mean voltage output values obtained for each Photodiode on Sensor 1 (orange) and Sensor 2 (blue).

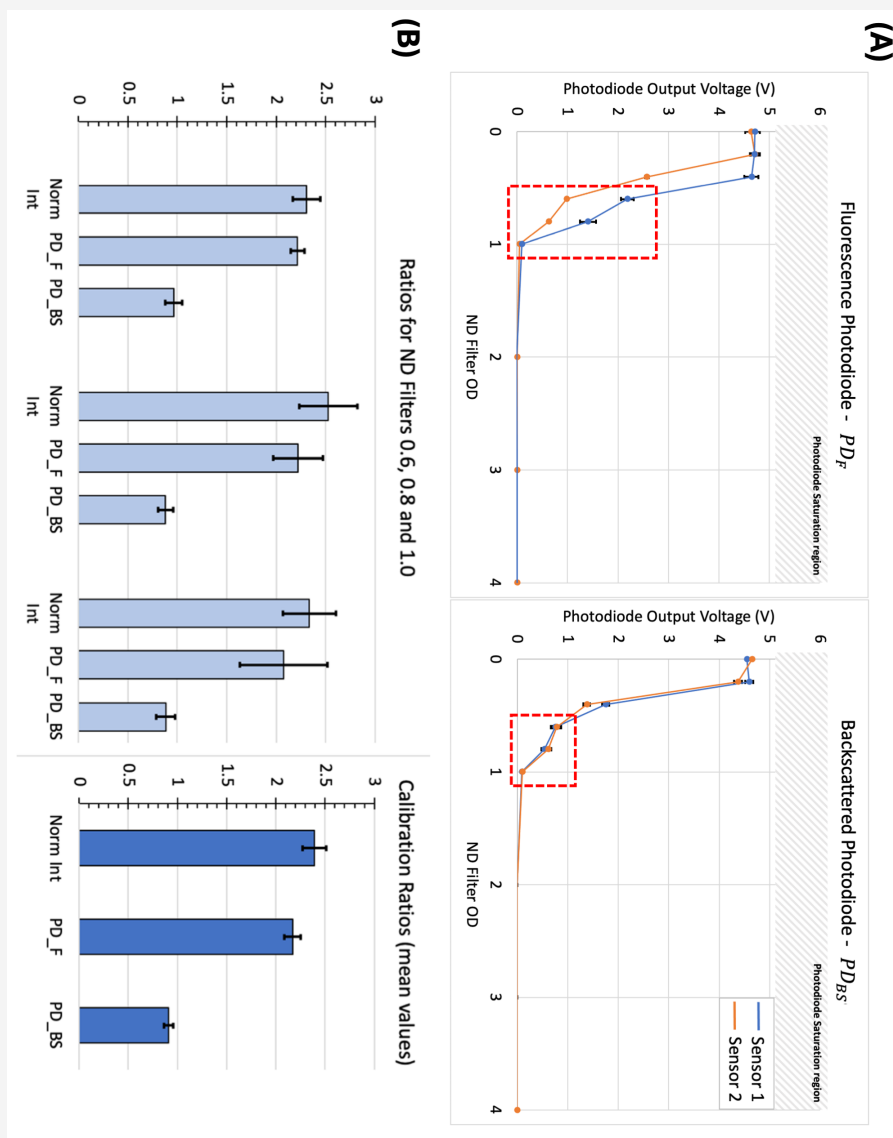


Figure 6.6: (A) Photodiode output signals from both of the sensors (Sensor 1 in blue and Sensor 2 in orange) for the different ND filters. Both photodiodes (Fluorescence photodiode in the left and Backscattered photodiode in the right) include a saturation region at a value of 5V, due to the configuration of the electronic circuit. Values used to calculate the Calibration Ratio are highlighted with a dotted red box, as they lie within the non-saturated and non-zero regions. These are the values from the 0.6, 0.8 and 1 ND filters. (B) Mean ratios and SD calculated from 10 repeats on 3 different days following equations 6.2, 6.3 and 6.4 for the output values from the 0.6, 0.8 and 1 ND Filters. (left) Individual ratios from each ND filter - 0.6 (left), 0.8 (middle) and 1.0 (right) - and Photodiodes, as well as the Normalised Intensity (Norm Int) values calculated following equation 3. The exact values can be observed in Table 6.4. (right) Final mean and SD Calibration ratios obtained for each photodiode as well as the Normalised Intensity are shown. These also correspond to the values presented in Table 3 - as the mean and SD values calculated across the 3 ND filters and the 3 days - and are used to correct for differences between the developed sensors (Sensor 1 and 2).

As expected, both sensors exhibit similar trends for both photodiodes. Three characteristic regions: saturation, transition zone and zero-region, can be observed. Internal sensors differences between Photodiodes are explained by the variations in the amplification gains used on each sensor (see Section 6.2.2.1). Following the analysis of the curves, we decided to use only the values from the transition regions (red box in Fig 6.6A) to calculate the Calibration factor. The reason for this is because values from the saturation or zero-regions do not encompass information about the differences between the sensors and can induce errors during the calculation of the calibration factor. Individual Fluorescence and Backscattered Photodiodes calibration factors, as well as a Normalized calibration factor were obtained following equations 6.2. The Normalized calibration coefficient was calculated following the same principle as the data normalization process explained previously in Chapter 5 and Ref [90]. Values for each ND filter, as well as the calibration coefficients can be seen in Table 6.4.

$$\text{Sensor 1} = \text{Sensor 2} * \text{Calibration ratio} \quad (6.2)$$

$$\text{Calibration ratio}_{PD_F, PD_{BS}} = \frac{\text{Sensor 1}_{PD_F, PD_{BS}}}{\text{Sensor 2}_{PD_F, PD_{BS}}} \quad (6.3)$$

$$\text{Calibration ratio}_{Norm} = \frac{\text{Calibration ratio}_{PD_F}}{\text{Calibration ratio}_{PD_{BS}}} \quad (6.4)$$

Table 6.4: Calibration output voltages from Sensor 1 and 2 using the ND filters. Output voltages from the photodiodes, as well as the calibration values calculated are shown. Only values from the transition regions (ND filter 0.6, 0.8 and 1.0) are shown for the Normalised Calibration ratios. Lower and higher values, corresponding to the saturation or zero-region, are not shown. Mean calibration ratios are calculated using the Calibration ratios from 0.6, 0.8 and 1, highlighted in bold, for each photodiode.

Output Voltage		Filter OD								
		0	0.2	0.4	0.6	0.8	1.0	2.0	3.0	4.0
PD _F	Sensor 1	4.715	4.703	4.646	2.184	1.404	0.096	-1.002*10 ⁻³	-1.352*10 ⁻⁴	-1.497*10 ⁻⁴
	Sensor 2	4.634	4.711	2.569	0.984	0.625	0.046	5.751*10 ⁻³	5.640*10 ⁻³	2.491*10 ⁻³
PD _{BS}	Sensor 1	4.549	4.599	1.752	0.753	0.529	0.091	-2.202*10 ⁻⁵	-1.189*10 ⁻⁵	-2.956*10 ⁻⁶
	Sensor 2	4.656	4.372	1.374	0.781	0.615	0.104	-5.735*10 ⁻⁵	-5.100*10 ⁻⁵	1.125*10 ⁻³

Calibration Ratio		Filter OD								
		0	0.2	0.4	0.6	0.8	1.0	2.0	3.0	4.0
PD _F		1.018	0.998	1.807	2.216	2.244	2.075	530.926	5.591	9.119
PD _{BS}		0.978	1.054	1.276	0.963	0.880	0.881	0.932	1.464	0.071
Normalised (PD _F / PD _{BS})					2.309	2.558	2.335			

Mean calibration Ratio	Mean	STD
PD _F	2.178	0.091
PD _{BS}	0.908	0.047
Normalised (PD _F / PD _{BS})	2.401	0.137

As shown in Table 6.4 and Fig. 6.6B, Calibration factors of 2.178 and 0.908 are obtained for the Fluorescence and Backscattered Photodiodes, respectively. These calibration factor obtained for both photodiodes (2.178 and 0.908) are in good agreement with the electronic differences between the sensors (see Section 6.2.2.1 and Fig. 6.2). These calibration factors ($R1 = 2.178$, $R2 = 0.908$ and $R_{Norm} = 2.401$) applied to Sensor 2 allow for direct comparison between dataset collected with both sensors and minimizes the impact of their electronic differences.

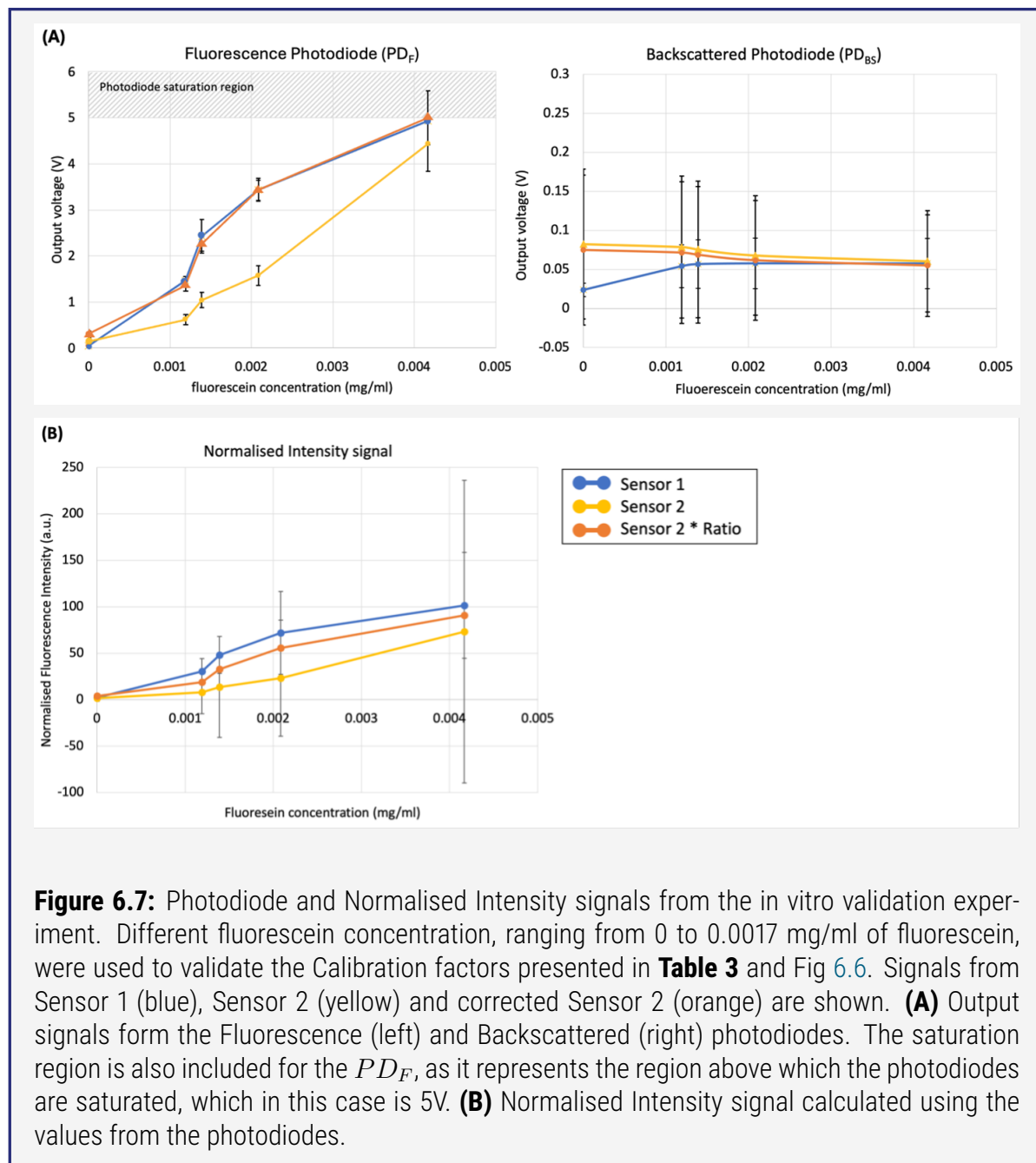


Figure 6.7: Photodiode and Normalised Intensity signals from the in vitro validation experiment. Different fluorescein concentration, ranging from 0 to 0.0017 mg/ml of fluorescein, were used to validate the Calibration factors presented in Table 3 and Fig 6.6. Signals from Sensor 1 (blue), Sensor 2 (yellow) and corrected Sensor 2 (orange) are shown. **(A)** Output signals from the Fluorescence (left) and Backscattered (right) photodiodes. The saturation region is also included for the PD_F , as it represents the region above which the photodiodes are saturated, which in this case is 5V. **(B)** Normalised Intensity signal calculated using the values from the photodiodes.

For the validation of the Calibration factor, fluorescence signals from both compact sensors were collected simultaneously using the different fluorescence solutions. Figure 6.7 shows the voltage values from the Fluorescence and Backscattered Photodiodes and the Normalised signals for Sen-

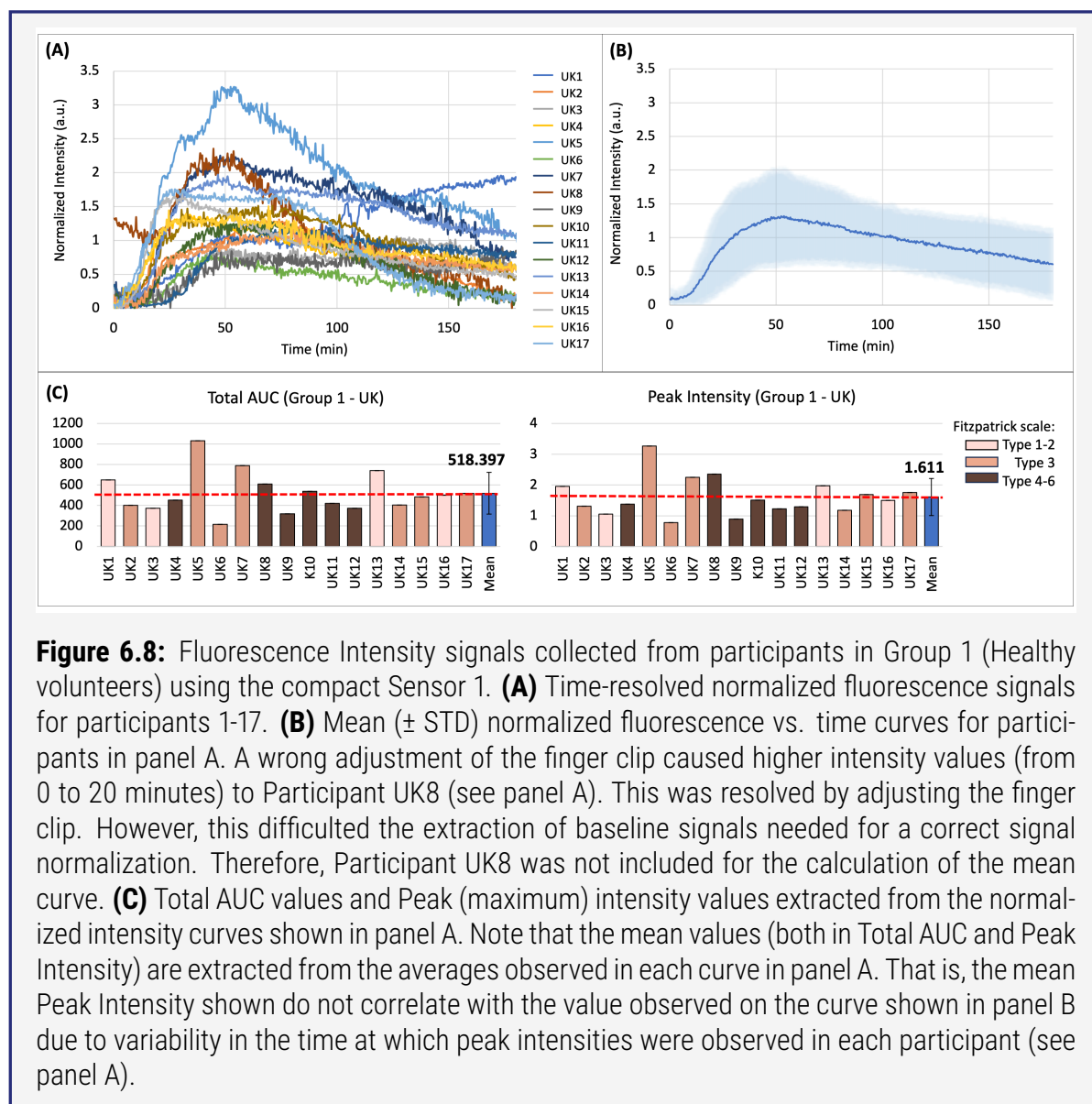
sensor 1 and Sensor 2. As predicted, output signals from the Backscattered Photodiodes (Fig. 6.7A) do not show any significant fluctuations, as the transparent plastic containers used for the fluorescence solutions do not produce any detectable light reflectance. However, as expected, the Fluorescence Photodiodes exhibit an increase in the output signals as the concentration of the solutions increased. The Fluorescence output signals observed for Sensor 1 were higher than that observed for Sensor 2 (Fig. 6.7B) due to the electronic differences described in Section 6.2.2.1. This is also noticeable when comparing the Normalised Intensity signals. Importantly, the differences in the fluorescence signals between sensors are compensated when the Calibration factors of 2.178 and 0.908 are applied to the Fluorescence and Backscattered signals from Sensor 2, respectively. The differences observed between the Normalised signals are primarily attributed to the Backscattered Photodiode signals, especially for the lower concentrations of fluorescein. This is because the material of the containers, although transparent, is a reflective surface that is likely to primarily impact the signals of the Backscattered Photodiodes, introducing variations between experiments and sensors. Nevertheless, the fluorescence signals, mainly affected by the changes in the electronic configuration, were successfully calibrated, allowing for a realistic comparison of datasets collected from both compact sensors, specifically relevant for clinical measurements. Subsequent sections, particularly Section 6.3.3 and 6.3.4, show signals from sensor 2 with the Calibration factors (PD_F and PD_{BS}) already applied.

6.3.2 Fluorescence intensity vs time - Healthy study

6.3.2.1 Fluorescence signal analysis

To investigate the potential of the compact fluorescence sensor – previously presented in Chapter 5 – as a portable tool for non-invasive assessment of gut function using transcutaneous fluorescence spectroscopy, 17 healthy volunteers (participants UK1-17) were recruited for an in vivo validation experiment in which the compact sensor (Sensor 1) was used to record fluorescence profiles. This data was also compared against the Lactulose: Mannitol permeability test – performed on the same participants – to analyze the correlation between these two procedures and to demonstrate the potential of the compact sensor for non-invasive assessment of gut permeability. Data from these 17 volunteers include signals from 6 volunteers previously recruited for chapter 5 who ingested a dose of 200 mg of fluorescein, as well as 11 additional volunteers recruited exclusively for this experiment.

All 17 participants were asked to fast for a minimum of 5 hours prior to the fluorescence spectroscopy experiment. At the start of the measurement, the fibre-optic probe from the compact Sensor 1 was attached to the participant's first fingers using a 3D-printed finger clip. Participants were asked to drink a fluorescein solution (200 mg fluorescein dissolved in 100 ml water), and fluorescence data was collected for 180 minutes. Fluorescence intensity vs. time curves from each participant is displayed in Fig. 6.8A.



As expected, most of the datasets exhibit an increase in fluorescence intensity over time, reaching a peak value approximately 50 minutes after ingestion of the fluorescein solution, before beginning to decrease back towards the background level (Fig. 6.8). It is important to mention that motion artifacts, caused by for example incorrect adjustment of the finger clip, can also affect the readings and induce errors. In Chapter 5, we have evaluated the impact of motion artifacts in the performance of the sensor and results suggested a non-significant impact on the signals. However, that evaluation only took into account the impact of motion artifacts when the finger clip was correctly attached to the finger. In cases where the finger clip is incorrectly attached or loose, detection of fluorescein signals is challenging. This was the case with Participant UK8 during the first 20 minutes of recording, in which the finger clip was placed incorrectly at the beginning of the experiment, leaving the fibre probe outside of the finger (non-skin contact). After adjusting the finger clip ($\approx$ 21 minutes) the sensor was able to detect the fluorescein permeating into the

bloodstream and the expected dynamic described before was observed. However, in this case, this motion artifact challenged the normalisation and noise correction of Participant UK8 signal as true baseline levels (collected at time 0 h) are not available. Therefore, in this case, this dataset (Participant UK8) was excluded for the analysis. However, it is reasonable to assert that in general, under the condition of no significant impact from motion or optical artifacts on the signals, differences in the fluorescence intensity profiles and decay rates between participants are likely attributed to inter-participant variability, such as different gastric emptying rates.

For further analysis, Total AUC and Peak Intensity values were also extracted from each participant (as shown in Fig. 6.8C). Data is color-coded to represent the Fitzpatrick score (previously presented in Chapter 5) assigned to each participant (i.e. Fitzpatrick Type 1/2, Type 3/4, or Type 5/6). As expected from previous studies, there is not a clear relationship between the normalized fluorescence intensity vs time curves generated by the compact sensor and the skin tone of the participants. This further supports the hypothesis presented in Chapter 5 that the performance of the compact sensor, and thus the fluorescence signal, is not adversely affected by changes in skin tone, and that differences in the observed fluorescence vs time curves are likely attributed to inter-participant variability.

Table 6.5: Parameters extracted from Group 1 using Sensor 1. Mean and std values are calculated from the individual values and not from the mean signal shown in Fig. 6.8. Values from Participant UK8 are only shown for informative purposes, and they are not used for the calculation of the mean values. Lactulose:Mannitol (LM) ratios and percentages of Lactulose (%L) and Mannitol (%M) are also included. L:M permeability parameters were not available for participants UK6, UK13 and UK15.

ID	Total AUC	Peak Int	Peak Time	Peak AUC	Peak AUC/ Slope	Peak AUC *Peak Time	%L	%M	LM ratio
UK1	647.962	1.956	173	594.885	58595.454	103113.57	0.299	15.071	0.0199
UK2	399.798	1.311	74	179.018	10340.847	13247.401	0.201	16.679	0.0121
UK3	371.823	1.057	126	233.879	36373.363	29624.749	0.346	21.023	0.0164
UK4	452.754	1.377	34	78.758	1765.022	2677.798	0.170	18.918	0.0090
UK5	1031.323	3.271	52	282.719	4042.835	14795.679	0.147	11.485	0.0128
UK6	215.303	0.775	41	36.568	1886.961	1511.502	-	-	-
UK7	788.615	2.251	47	156.531	2739.857	7461.292	0.055	13.988	0.0039
UK8	607.884	2.356	44	200.932	7232.329	8974.992	0.301	28.809	0.0105
UK9	317.42	0.892	174	294.702	110715.817	51278.258	0.150	11.815	0.0127
UK10	537.911	1.511	71	215.72	10407.111	15316.135	0.099	11.675	0.0084
UK11	420.39	1.225	80	143.426	8086.627	11521.901	0.140	25.172	0.0055
UK12	370.996	1.288	61	132.324	5571.689	8071.811	0.186	30.757	0.0061
UK13	741.229	1.973	46	142.771	2821.515	6662.643	-	-	-
UK14	404.873	1.181	80	177.666	12980.043	14272.545	0.105	31.160	0.0034
UK15	484.334	1.687	30	80.912	1149.392	2481.313	-	-	-
UK16	501.114	1.504	69	214.303	12740.416	14858.376	0.239	22.503	0.0106
UK17	519.023	1.757	31	94.578	1407.103	2963.466	0.233	20.356	0.0114
Mean	512.804	1.563	74.625	191.173	17601.503	18741.152	0.182	19.276	0.0102
STD	203.343	0.607	45.599	129.791	29145.27	25684.797	0.082	6.764	0.0048

Following the analysis, the Total AUC mean value, calculated using the individual curves, was 512.804 (a.u.), with a standard deviation of 203.343 (39.653%), and the maximum Peak Intensity mean value was 1.563 (a.u.), with a standard deviation of 0.607 (38.835%). Parameters, such as Peak AUC/Slope and Peak AUC*Peak Time were also calculated in order to correct for the impact of gastric emptying or gut motility (as explained in chapter 4). These values, together with the individual Total AUC values and other extracted parameters (such as the Peak Time, Peak Intensity and Peak AUC) for all individual signals are shown in Table 6.5.

6.3.2.2 Lactulose:Mannitol correlation

To further validate the performance of the compact fibre-optic sensor, a Lactulose: Mannitol (L:M) test was carried out by all participants. At least one week after the fluorescence spectroscopy experiment, all participants were asked to undertake a L:M permeability test. All 17 participants were asked to fast for a minimum of 5 hours prior to the permeability test and continue fasting throughout the 6 hours experiment. Before starting the test, urine samples were collected and labelled as “baseline”. Participants were then asked to drink a sugar solution containing 10 g of Lactulose and 5 g of Mannitol diluted in 100 ml of water, together with 300 ml of water. After 3 hours from the start of the experiment, participants were able to drink an additional 200 ml of water. Urine samples were collected during a period of 6 hours. At the end of the experiment, total urine volume was measured and samples of 1ml aliquots were analysed. Percentage excretion of Lactulose and Mannitol, as well as Lactulose:Mannitol Ratios (L:MR), for each participant are shown in Table 6.5.

The mean percentage excretion of Lactulose and Mannitol in participants from Group 1 are 0.182% (range 0.055 – 0.345) and 19.276% (range 11.484 – 31.160), respectively. The mean L:M ratio observed among the participants is 0.0102 (range 0.003 – 0.0198), a value which is consistent with published data for healthy subjects [130]. Although, participant UK1 exhibits the highest L:M ratio (0.0198), in comparison with participants UK7 and UK14 with the lowest L:M ratios (0.0039 and 0.0033), all the participants L:M ratios lie within the range of healthy subjects, as previously published in [130]. Generally, lower L:M ratios are considered indicative of better intestinal barrier function [130, 142]. However, L:M ratios alone do not necessary diagnose a permeability problem because, although L:M test provide valuable insights about the gut permeability, clinical correlation with other markers and symptoms is crucial for a comprehensive evaluation of the gut barrier.

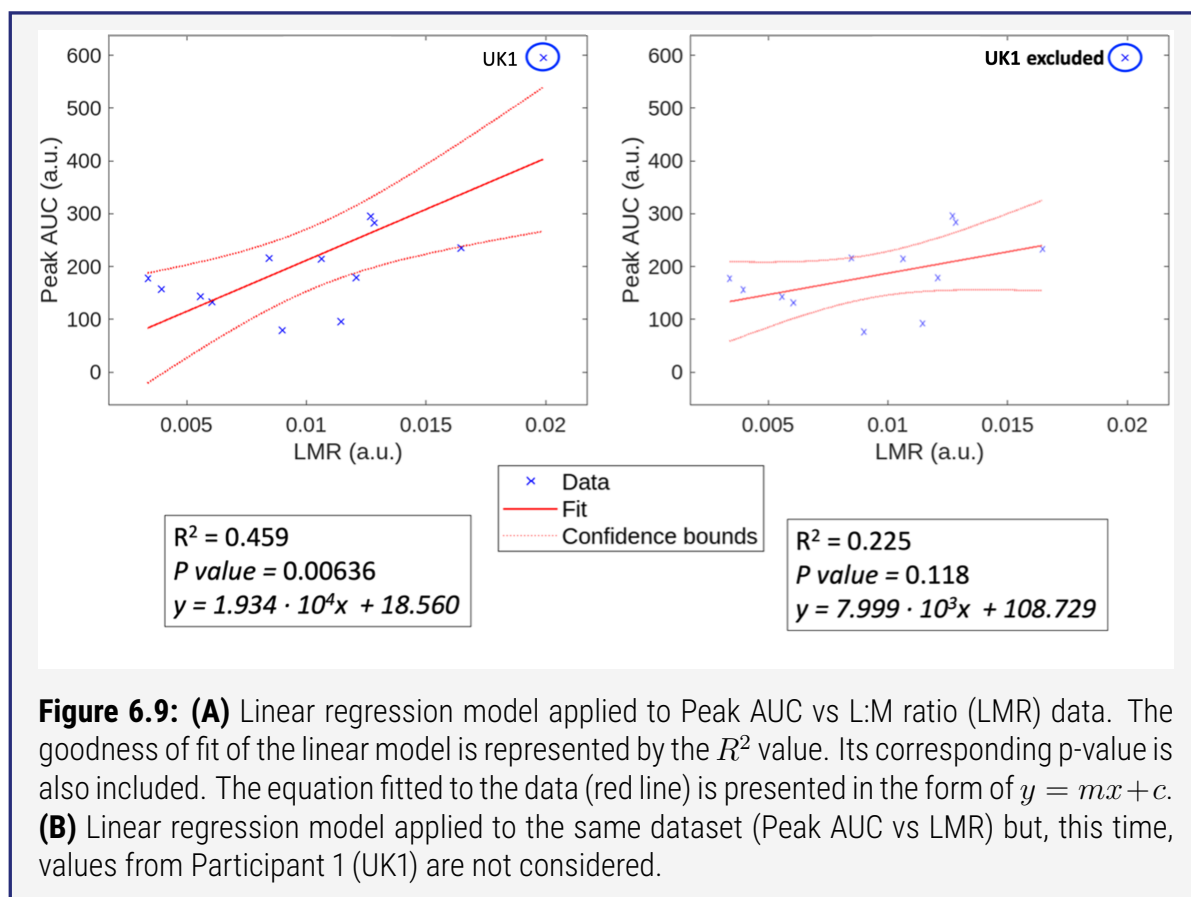
For the value of this research, parameters extracted from the L:M permeability test are mainly used to analyse the correlation between the fluorescence intensity signal collected with the compact sensors and the L:M parameters. To do so, Pearson's correlation coefficients were calculated using the parameters extracted from the fluorescence intensity signals from Sensor 1 (Total AUC, Peak AUC, Peak Intensity, Peak AUC/Slope and Peak AUC*Peak Time values) and L:M results (%L, %

M and L:MR) of each participant. Table 6.6 shows the Pearson's correlation coefficient (r) of each parameter analysed, as well as the p-values. Values from Participants UK13 and UK15 are not considered for this analysis, as L:M parameters were not available.

As shown in Table 6.6, some Pearson's correlation coefficients (r) are < 0 , suggesting a negative correlation between both approaches. Peak AUC vs L:MR and Total AUC vs L:MR show highest and lowest correlations, respectively. This is also supported by the p-values extracted from the analysis (0.0064 and 0.7462, respectively). In addition, a linear regression model was fit to the data with highest correlation (i.e., Peak AUC vs L:MR), with $R^2 = 0.459$ and p-value = 0.00636 (i.e., for the equation $y = mx + c$, where m ($m = 19338$) represents the gradient and c ($c = 18.56$) represents the intercept), see Fig. 6.9. It is worth noting that the correlation described before between the Peak AUC and L:MR, is significantly impacted by the presence of an outlier (labelled as P1 in Fig. 6.9). When removing this data point (Fig. 6.9(right)), the correlation between these two parameters, although moderate (with a Pearson's correlation coefficient of 0.474), is no longer significant (p value = 0.118).

Table 6.6: Pearson's correlation coefficients (r) and p-values extracted from the correlation between Sensor 1 (Total AUC, Peak AUC, Peak Intensity Peak AUC/Slope and Peak AUC*Peak Time) and Lactulose:Mannitol test (percentage of Lactulose (%L) and Mannitol (%M) and LM ratio (LMR)). Significant correlations are highlighted in bold.

Pearson's correlation coefficient (r)					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
%L	-0.2364	-0.1801	0.3933	0.2437	0.4528
%M	-0.5072	-0.4525	-0.3931	-0.3347	-0.3098
LMR	0.0996	0.1251	0.7117	0.5082	0.7102
P values from the Pearson's correlation coefficients					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
%L	0.4369	0.5559	0.1837	0.4224	0.1202
%M	0.0768	0.1205	0.1839	0.2637	0.3029
LMR	0.7462	0.6838	0.0064	0.0762	0.0065



6.3.2.3 Summary

An increased gut permeation is expected to correlate with an increased translocation of both sugar molecules and fluorescein. Consistent with this idea, the results presented in this section suggest a moderate correlation between the compact sensor (Sensor 1) and the L:M permeability test. However, the correlation between these two techniques proved to be significantly impacted by the presence or absence of specific points (outliers). This could be attributed to the limited data available and the complexity of it. In addition, as explained previously, both experiments were undertaken at different periods in time, with generally a time gap of more than 1 week between data collection for Sensor 1 and L:M urine samples. Over this time period, variations in diet, temporary gut disorders (such as diarrhoea or constipation) or medication, can significantly affect gut permeability and therefore, the correlation between both approaches. In order to mitigate this effect and to characterise more effectively the correlation between these two tests, permeability tests, such as L:M or L:R, and fluorescence intensity signals from the compact sensor are recommended to be carried out simultaneously. This has, therefore, been considered in the following section. In addition, this analysis will also benefit from recording information regarding dietary habits, medications or any other confounding factors.

6.3.3 Fluorescence intensity vs time - Undernutrition study

6.3.3.1 Fluorescence signal analysis

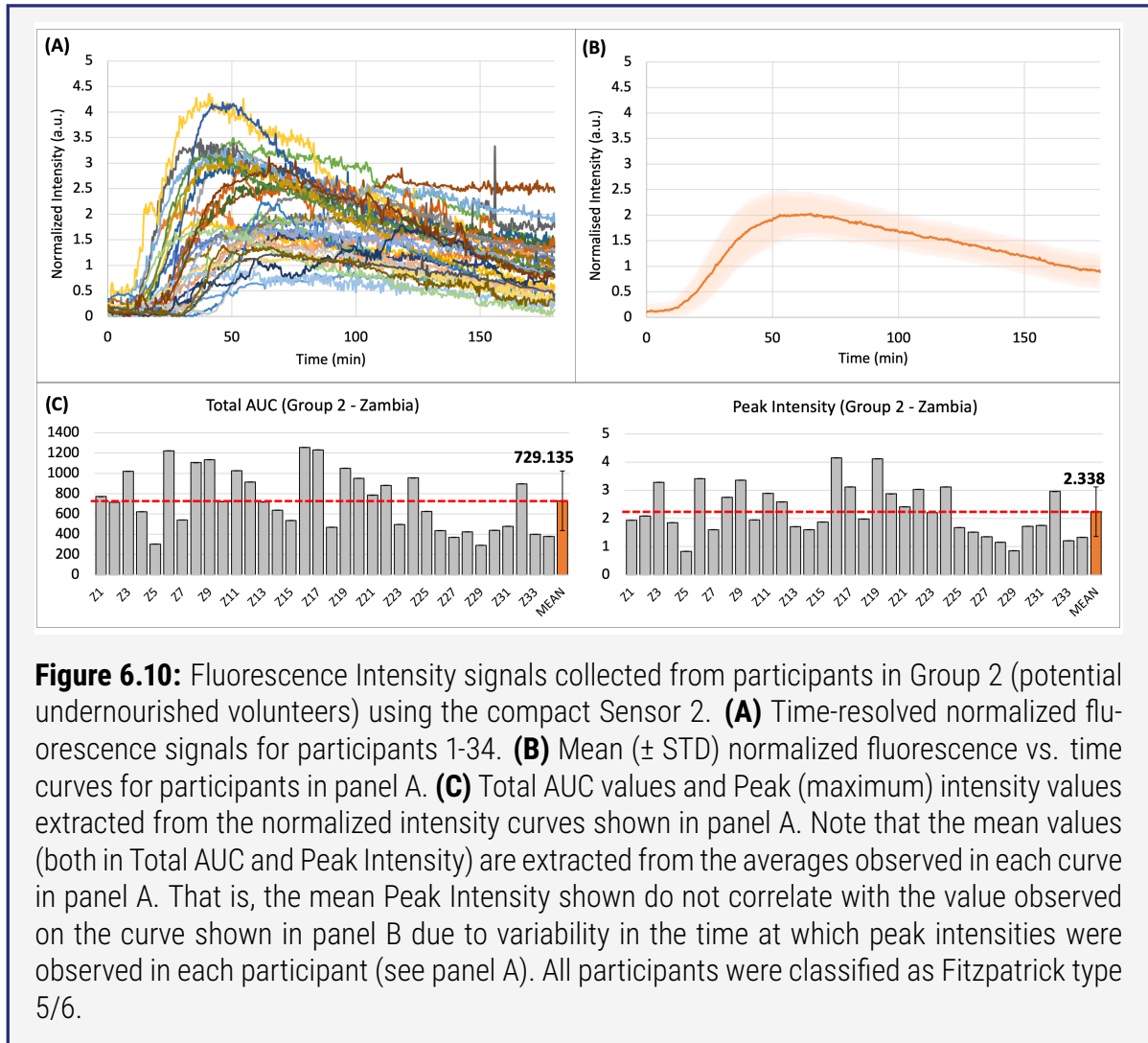
Following the deployment of Sensor 1 in St Mary's Hospital (UK) for the collection and analysis of fluorescence signals on healthy volunteer (Section 6.2.2.2), we focused on investigating the potential of the developed Sensor 2 as a portable tool for non-invasive assessment of gut function in potential undernutrition participants. In this case, 80 volunteers were recruited for an in vivo validation experiment in which the compact sensor (Sensor 2) was used to record fluorescence profiles from 34 participants (participants Z1-34).

Following the same procedure as described in Section (Section 6.2.2.2), the data collected from this group was also compared against the Lactulose: Rhamnose permeability test – performed on the same participants alongside fluorescein detection. The objective is to analyse the correlation between the fluorescence intensity data (this time from Sensor 2) and the permeability assay (L:R test) and to further demonstrate the potential of the compact sensor for non-invasive assessment of gut permeability.

Following a similar protocol as the one detailed in Section (Section 6.2.2.2), all 34 participants underwent a fasting period of at least 5 hours prior to the fluorescence spectroscopy experiment. At the start of the experiment, the fibre-optic probe from Sensor 2 was attached and secured to the participants' index finger using the 3D-printed finger clip depicted in Fig. 6.1C. Following this, participants were asked to drink a fluorescein solution and fluorescence intensity vs time curves were collected for a duration of 180 minutes. It is important to mention that the ingested fluorescein solution also contained the sugar molecules for the L:R test, which is later discussed. Therefore, the final ingested fluorescein solution contains 200 mg of fluorescein, 5 g of Lactulose and 1 g of Rhamnose diluted in 200 ml of water. Figure 6.10 illustrates the fluorescence intensity data collected from each participant.

As previously observed in the Group 1 (Section 6.2.2.2), the majority of datasets in the current study demonstrate an upward trend in fluorescence intensity over the 180-minute of data collection period. Generally, peak intensity values are reached approximately 70 minutes post-ingestion of the fluorescein solution, followed by a subsequent decline towards the baseline level (refer to Fig. 6.10).

Following the analysis of the fluorescence vs time curves, Total AUC, as well as other parameters (Peak Intensity, Peak Time, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time) were extracted from the signals (Table 6.7). The individual, as well as mean values of the Total AUC and Peak Intensity parameters are shown in Fig. 6.10C. Total AUC mean value, calculated using the individual curves, was 729.135 (a.u.), with a standard deviation of 293.207 (40.212%), and the maximum Peak Intensity mean value was 2.338 (a.u.), with a standard deviation of 0.896 (38.232%).



In this case, individual values are not color-coded due to the skin homogeneity of the group (all 34 participants present Fitzpatrick's type 5-6). Previous studies have shown that darker skin types (Fitzpatrick's 5-6 types) exhibit higher epidermal melanin concentrations than lighter skin types (Fitzpatrick's 1-3 types), producing differences in absorption and scattering profiles that could potentially impact the sensitivity of the developed compact sensors [240]. However, as anticipated from previous experiments, including findings from Chapter 5 and Section 6.3.2.1, there is not substantial correlation between the normalized fluorescence intensity signals and the Fitzpatrick scores. These outcomes suggest, once more, that strategies implemented in the sensors effectively address and rectify variations in the absorption and scattering profiles of diverse skin tones. Therefore, differences in the observed fluorescence vs time curves are likely produced by inter-participant variability, such as gastric emptying rates.

Table 6.7: Parameters extracted from Group 2 using Sensor 2. Mean and std values are calculated from the individual values and not from the mean signal shown in Fig. 6.10. Lactulose:Mannitol (LM) ratios and percentages of Lactulose (%L) and Rhamnose (%R) are also included. Null values (labelled as "-"), correspond to unavailable data.

ID	Total AUC	Peak Intensity	Peak Time	Peak AUC	Peak AUC/ Slope	Peak AUC* Peak Time	%L	%R	LR ratio
Z2	771.566	2.086	95	390.733	15002	28737.674	0.065	0.253	0.259
Z3	715.383	2.149	40	148.512	2988.8	6299.497	0.131	-	-
Z4	1020.342	3.291	48	145.041	2380.8	7837.068	1.329	12.070	0.110
Z5	622.175	1.895	38	93.194	1955	3493.954	0.065	-	-
Z7	302.671	1.052	119	185.698	26185	21575.149	0.023	0.623	0.037
Z8	1222.488	3.491	50	248.846	3943.7	13463.278	-	-	-
Z9	540.058	1.678	77	182.678	10997	17555.826	-	-	-
Z10	1105.918	2.901	118	635.637	26855	71664.989	-	-	-
Z11	1134.237	3.463	37	181.413	2004	6643.987	-	0.248	-
Z12	722.143	2.111	91	327.087	12182	23745.884	-	-	-
Z13	1026.211	3.019	73	409.241	2996.1	8606.254	-	-	-
Z14	913.892	2.675	72	298.479	8459.8	21863.586	-	-	-
Z15	717.742	1.738	54	131.737	7676.6	12996.513	-	-	-
Z16	636.684	1.646	131	432.528	28771	46064.785	-	-	-
Z17	534.301	1.986	86	268.846	8653	15752.383	-	-	-
Z18	1256.022	4.353	40	280.905	2745.5	11492.53	-	-	-
Z19	1231.354	3.296	47	258.695	6063.3	19008.895	-	-	-
Z20	468.528	2.042	61	109.023	3252.7	6429.938	-	-	-
Z21	1049.534	4.193	46	199.939	2968.8	12311.042	-	-	-
Z22	951.076	3.041	53	175.521	3254.7	9107.232	-	-	-
Z23	784.373	2.501	79	220.378	9194.5	22028.587	-	-	-
Z24	882.168	3.171	49	234.035	3692.2	11261.964	-	-	-
Z25	495.642	2.239	62	84.598	2865.4	5950.252	-	1.145	-
Z26	955.296	3.174	38	143.437	1744.2	5494.868	-	-	-
Z27	624.717	1.752	83	243.585	6892.1	11582.493	-	-	-
Z70	434.615	1.575	60	94.171	3711.9	5431.818	0.093	2.414	0.038
Z71	368.945	1.391	86	145.817	6295.5	8272.372	0.194	3.289	0.059
Z72	423.062	1.212	86	212.116	15434	17948.346	0.141	2.558	0.055
Z73	291.321	0.931	79	140.771	5431.4	4620.971	0.084	1.791	0.047
Z74	438.035	1.921	36	94.759	3604	6237.123	0.369	-	-
Z75	477.527	1.786	118	265.363	17974	30956.536	0.347	3.347	0.104
Z77	895.588	3.125	65	265.888	6276.2	18472.876	0.328	7.295	0.045
Z78	399.902	1.218	66	94.574	5149.2	5832.684	0.158	2.010	0.079
Z79	377.082	1.393	46	41.253	1381.1	1774.232	0.574	12.635	0.045
Mean	729.135	2.338	68	217.191	7911.19118	15309.282	0.279	3.821	0.079
STD	293.207	0.896	26.376	120.877	7405.14798	13660.981	0.339	4.205	0.065

6.3.3.2 Lactulose:Rhamnose correlation

Similar to Section 6.3.2.2 (Group 1), further validation of the performance of the compact sensor focused on evaluating the correlation with Lactulose: Rhamnose ratios (LRR) collected from each participant in Group 2. In this case, all 80 participants ingested the L:R solution at the same time as the fluorescein solution and urine samples were collected on the same day as the fluorescein intensity signals. As before, all participants were asked to fast for a minimum of 5 hours prior to the permeability test and continue fasting throughout the measurements. Before starting the test, urine samples were also collected and labelled as "baseline". Because the permeability test

(L:R test) was conducted at the same time as the fluorescein experiment, participants were asked to drink a final solution, containing, in this case, 200 mg of fluorescein, 5 g of Lactulose and 1g of Rhamnose diluted in 200 ml of water. Urine samples were collected after a period of 3 hours. Total urine volume was quantified and 1ml aliquots samples were stored for further analysis at the same facilities as the L:M samples. Lactulose: Rhamnose Ratios (LRR) for each participant are shown in Table 6.7. It is important to note that the analysis is undertaken using data from only 34 participants (those with fluorescence signals available) out of the 80 that were originally recruited. In addition, out of these 34 participants, only 41% of the participants (n=14) had L:R data available for this analysis. This is due to limited amount of Lactulose available during the experiment, leading to only a subset of participants conducting the L:R test.

The mean percentage excretion of Lactulose (%L) and Rhamnose (%R) for Group 2 are 0.278% (range 0.023 – 1.328) and 3.087% (range 0.247 – 12.634), respectively. The mean L:R ratio observed among the participants is 0.080 (range 0.037 – 0.259), a value higher than those reported in the literature for healthy subjects (range 0.0018 – 0.0035) [134].

Taking into account the results from the L:R assay, participant Z79 exhibits the highest percentage of Lactulose recovered, which based on the literature, it could indicate an abnormal permeability [130, 142]. This could also be applicable to Participant Z1, exhibiting the highest L:R ratio (0.259). However, assessing the stage of gut permeability using these values alone, including the L:R ratio, is subject to misinterpretations due to the challenges associated with the lack of standardisation of the protocols used in the various studies [130, 142]. In addition, as explained previously, L:R (or L:M) values alone do not necessary indicate a permeability problem and correlation with other markers and symptoms is necessary for a complete understanding of the gut permeability condition.

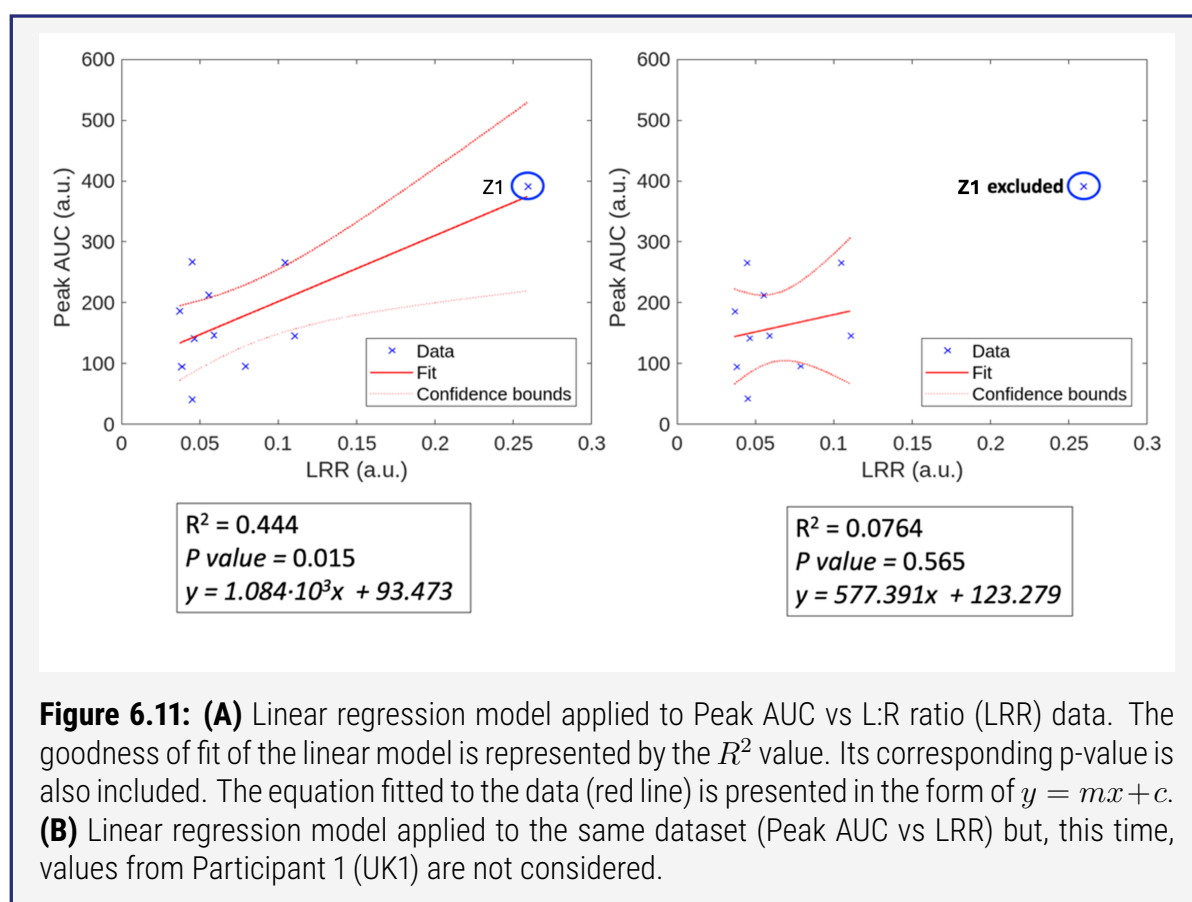
Table 6.8: Pearson's correlation coefficients (r) and p-values extracted from the correlation between Sensor 2 (Total AUC, Peak AUC, Peak Intensity Peak AUC/Slope and Peak AUC*Peak Time) and Lactulose:Rhamnose test (percentage of Lactulose (%L) and Rhamnose (%R) and LR ratio (LRR)). Significant correlations are highlighted in bold.

Pearson's correlation coefficient (r)					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
%L	0.5389	0.6299	-0.01618 -0.3326	-0.1575	
%R	0.1989	0.2634	-0.3031	-0.4009	-0.2768
LRR	0.4759	0.2057	0.7069	0.1873	0.5306
P values from the Pearson's correlation coefficients					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
%L	0.0467	0.0158	0.5806	0.2452	0.5907
%R	0.5146	0.3847	0.3139	0.1745	0.3599
LRR	0.1389	0.3607	0.0149	0.5814	0.0931

Following the same procedure as for L:M samples from Group 1, correlation between the compact sensor and the L:R test results (%L, %R and LRR) was calculated using the Pearson's correlation

coefficient (r), see Table 6.8. It is, however, worth noting that the limited L:M data available ($n=14$ for %L and %R; and $n=11$ for LRR) inevitably constrains the depth of the analysis and, subsequently, the interpretation of the results. Comparison between the fluorescence parameters and the L:R values is, therefore, conducted with this limitation in mind.

Similar to the observations in Section 6.3.2 (for Group 1), Peak AUC vs LRR exhibits the highest correlation ($r=0.7069$), with p value = 0.0149. However, as before, the significance of this correlation is highly impacted by the LRR value of Participant Z1 (see Fig. 6.11). When removing this data point from the analysis, correlation coefficient (r) is reduced to 0.2078, with a p value = 0.5647. Figure 6.11 displays the linear regression model applied to the data in both cases (i.e., with and without data point Z1), as well as the R^2 and p -values obtained from the regression model.



Total AUC and Peak Intensity also exhibit significant positive correlation with the percentage of Lactulose (%L). However, when values from Participant Z4 are removed from the analysis, correlations are no longer significant ($r < 0.2448$ and p -values > 0.05). This high dependence is, therefore, primarily attributed to the limited data available ($n < 14$), and so for the purposes of the thesis, the interpretation of the results is based on the correlations calculated using all the data available in each case (i.e., including the potential “outliers”). Therefore, the significant correlations observed between the fluorescence signals and the %L would be associated to the similar molecular weight of Lactulose and Fluorescein (342.299 and 332.319 mg/ml, respectively). It is then expected that

both molecules follow similar permeability pathways, thereby characterising similar aspects of the gut barrier integrity. In addition, results presented by Ordiz et al. would support the idea that a single molecular marker such as Lactulose or fluorescein (in our case) could potentially give similar information as dual sugar permeability assays, such as L:M or L:R tests [125].

Higher %L values are commonly associated with increased gut permeability [130, 142], which, as shown, correlates with higher rate of fluorescein translocation (i.e. higher fluorescence intensity signals). Overall, the results presented here suggest, again, a moderate linear correlation between the fluorescence compact approach and the L:R permeability test. However, it is important to highlight the limitations associated with data availability and the challenges inherent in interpreting the L:R results. This is the reason why in this thesis we focus exclusively in analysing the correlation between these two approaches. Considering this, the complexity of interpreting the correlation between both approaches, including the L:M test, extends to the potential impact that other confounding factors, including diet and medication, could have on the parameters here analysed. However, the fact that, in this case, both approaches were carried out simultaneously suggest that these confounding factors would have affected both approaches similarly. This is not the case in Section 6.3.2, where L:M test was performed at least one week after the fluorescence test and confounding factors might have a bigger impact in the correlation between the approaches.

Nevertheless, the results presented here show the potential use of the fluorescence sensor to assess gut permeability. In addition, the need for a laboratory-based facility to process and analyse the L:M and L:R parameters, together with associated cost and time requirements, underscores the pressing need for improved technologies. This includes, for example the use of portable optical sensors, such as the one developed in this thesis, to assess gut permeability. However, further investigation of the correlation of the fluorescence signals with other physiology parameters, will provide more information about the functionality of this non-invasive fluorescence approach.

6.3.3.3 Morphometry analysis

Following this idea, duodenal biopsies were provided by the team in Zambia, from participants in Group 2, to correlate with the fluorescence parameters extracted from the signals using Sensor 2. The morphology parameter will also help to further understand the physiology of the gastrointestinal wall of the participants involved in Group 2. Parameters, such as villus height (VH), crypt depth (CD), villus-to-crypt ratio (VCR) and epithelium surface area (ESA), were extracted, by the team of experts in Lusaka, from the samples for each participant. Table 6.9 shows the values for each parameter and participant. Previous studies have shown that villus atrophy (flattened epithelial barrier) is associated with reduced uptake of nutrients that pass through the gastrointestinal tract [241–243]. The intestinal integrity is, then, generally assessed using the villus-to-crypt ratio (VCR), which takes the VH and CD into account [96, 244]. However, normal VCR value is subject to some controversy.

Table 6.9: Morphometry parameters extracted from the duodenal biopsies from Group 2.

Parameters include villus height (VH), crypt depth (CD) epithelium surface area (ESA) and villus-crypt ratio (VCR). Only values from the 34 participants with available fluorescence signals are used for the analysis. In addition to this, zero /null values for participants Z4-7, Z22-23 and Z78-79 correspond to unavailable biopsies. Units for VH and CD are micrometres (μm), whereas ESA is expressed in square micrometres (μm^2).

ID	VH	CD	ESA	VC Ratio
Z2	204.409	129.682	509.029	1.576
Z3	184.282	110.026	601.849	1.675
Z4	-	-	-	-
Z5	-	-	-	-
Z7	-	-	-	-
Z8	174.143	250.714	375.775	0.695
Z9	199	136.4	471.295	1.459
Z10	245.4	142.6	429.34	1.721
Z11	265	122.5	357.02	2.163
Z12	254.167	182.75	768.961	1.391
Z13	158	199.5	232.077	0.792
Z14	217.667	147.267	501.944	1.478
Z15	231.87	120.174	673.3	1.929
Z16	222.2	102.4	412.263	2.17
Z17	290.125	166.063	479.745	1.747
Z18	227.714	140.857	444.614	1.617
Z19	244.222	123.889	533.373	1.971
Z20	211	137	219.942	1.54
Z21	202	186.2	314.199	1.085
Z22	-	-	-	-
Z23	-	-	-	-
Z24	189.313	133.375	542.185	1.419
Z25	181.5	90.857	402.421	1.998
Z26	169.4	107.4	451.402	1.577
Z27	167.1	99.3	490.598	1.683
Z70	109.333	144	348.282	0.759
Z71	166.5	203.5	335.321	0.818
Z72	210.222	128.778	442.496	1.632
Z73	205.429	154.429	423.525	1.33
Z74	222.538	122.308	632.35	1.819
Z75	149.867	141.333	448.911	1.06
Z77	186.25	212.25	585.763	0.878
Z78	-	-	-	-
Z79	-	-	-	-
Mean	203.283	145.761	460.296	1.481

Generally, normal **VCR** range is associated with values between 3:1 to 5:1 [241]. However, other ratios, such as 2:1 or 1:1 have also been reported [245]. Another parameter analysed is the Epithelium surface area (**ESA**). It is a direct measurement of the surface of the epithelial cells within, in this case, the duodenum. Previous studies have found changes in the epithelial surface area, **VH** and **CD** associated with patients with malabsorption disorders, including Crohn's disease and malnutrition [242, 244, 246].

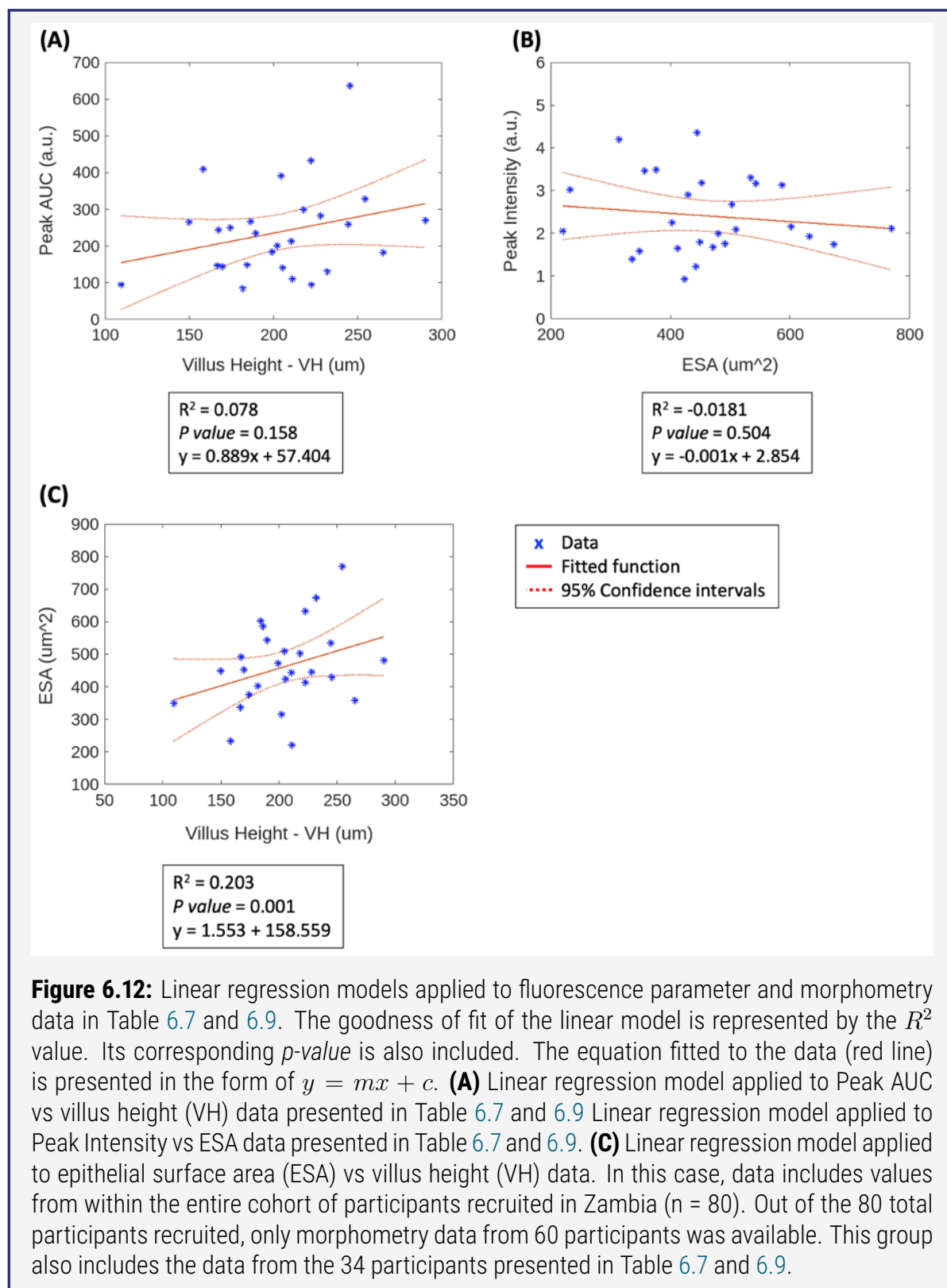
Therefore, we further characterize the functionality of the fluorescence sensor (in this case, Sensor 2) and the potential of the fluorescence parameters to assess changes in gut permeability. To do so, we calculate the correlation between the morphology parameters (**VH**, **CD**, **VCR** and **ESA**) and the fluorescence intensity signals using the Pearson's correlation coefficient. Table 6.10 shows the results from this analysis. It is important to mention that considering the controversy associated with the **VCR** values, this analysis focuses on the correlation of the fluorescence signals with the morphometry parameters, without any data diagnostic purposes.

Table 6.10: Pearson's correlation coefficients (r) and p-values extracted from the correlation between Sensor 2 (Total AUC, Peak AUC, Peak Intensity Peak AUC/Slope and Peak AUC*Peak Time) and morphometry parameters (villus height (VH), Crypt depth (CD), epithelial surface area (ESA)) and Villus-to-Crypt ratio (VCR). None of the correlations were found statistically significant.

Pearson's correlation coefficient (r)					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
VH	0.2354	0.1517	0.2793	0.2241	0.2902
CD	0.2317	0.2597	0.1646	-0.1489	-0.0382
ESA	-0.0238	-0.1345	0.0087	0.1195	0.1100
VCR	0.0254	-0.0647	0.0364	0.2318	0.1875
P values from the Pearson's correlation coefficients					
Parameters	Total AUC	Peak Intensity	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
VH	0.2371	0.4499	0.1583	0.2612	0.1420
CD	0.2448	0.1908	0.4120	0.4584	0.8501
ESA	0.9064	0.5037	0.9657	0.5527	0.5848
VCR	0.8999	0.7486	0.8571	0.2446	0.3489

Values from Participants Z4-7, Z22-23 and Z78-79 are not considered in this case, as biopsy parameters were not available (see Table 6.9). As shown in Table 6.10, no significant correlations were found in any of the cases. As previously explained, flattened epithelium (i.e., low VH and CD) are linked to gut barrier dysfunction and nutrient malabsorption. Therefore, opposite to what we expected, fluorescence parameters exhibit a subtle positive correlation with the **VH** and **CD** values, with the highest Pearson's correlation coefficients found between the Peak **AUC** *Peak Time parameter and the **VH** ($r = 0.2902$). In the case of **ESA**, a negative correlation is observed for both Total **AUC** and Peak Intensity, which is consistent with the idea that lower **ESA** (i.e., weakened epithelium) is associated with barrier dysfunction. The results presented in Table 6.10 are also supported

by the linear regression model fitted to the data. Fig. 6.12A-B shows the linear regression model applied to Peak AUC vs VH and Peak Intensity vs ESA.



The results from table 6.9 and Fig 6.12, specially for Peak Intensity, could suggest that lower epithelial surface area (ESA), common in disrupted gut barriers, is associated with an increase in the

permeation of fluorescein. This is observed, for example, in patients with coeliac disease [247]. In addition, during mucosal atrophy, with an increase in intestinal dysfunction, changes in epithelial barrier are characterised by decreased **VH**, **CD**, as well as **ESA** [242, 247]. This would explain the positive correlation observed when comparing the **VH** and **ESA** parameters from participants in Group 2 (see Fig. 6.12C). However, although promising, these results do not show enough evidence to establish a solid correlation between fluorescence permeation and the morphometry data.

Similar results were observed when analysing the correlation between the **L:R** and the morphometry parameters (Table 6.11). Non-significant correlation was found when analysing these two approaches, with the highest Pearson's correlation coefficient (0.2569; *p* value = 0.1629) found between the Villus-to-Crypt ratio (**VCR**) and the % Lactulose (% L). It is important to note that the correlation analysis in this instance was conducted using data from 36 participants out of the total 80, where **L:R** and morphometry parameters were simultaneously available. The higher number of data points available for this analysis, in comparison with the previous correlation, suggests that these results may reflect the true lack of correlation between these two clinically established approaches.

Table 6.11: Pearson's correlation coefficients (r) and p-values extracted from the correlation between the **L:R** test (%L, %R and LRR) and the morphometry parameters (villus height (**VH**), Crypt depth (**CD**), epithelial surface area (**ESA**)) and Villus-to-Crypt ratio (**VCR**). None of the correlations were found statistically significant.

Pearson's correlation coefficient (<i>r</i>)				
Parameters	VH	CD	ESA	VCR
%L	0.1099	-0.0854	-0.1492	0.2569
%R	0.1271	-0.0441	-0.0119	0.2048
LRR	-0.1137	0.0516	0.0229	0.1413
<i>P</i> values from the Pearson's correlation coefficients				
Parameters	VH	CD	ESA	VCR
%L	0.5558	0.6479	0.4231	0.1629
%R	0.4957	0.8136	0.9491	0.2689
LRR	0.5802	0.8025	0.9118	0.4909

6.3.3.4 Summary

Literature studies have shown that a reduction in **VH** is often associated with increased permeability. This would theoretically lead to an increase in the translocation of molecules such as Lactulose or fluorescein, resulting in higher **L:R** and fluorescence parameter values, respectively. The multiple changes occurring in the epithelial barrier have been commonly assessed using the morphometry

parameters presented before [242, 244, 247]. However, the results from this section would support the idea that the small representation of the biopsies is unlikely to represent all the changes that occur across the very large surface of the small intestine. For example, Kelly et al. showed that changes in the surface area (ESA) could also be attributed to changes in the surface area of the microvillus, which are significantly more complex to assess [244].

The lack of significant correlation between the morphometry data and the fluorescence parameters does not invalidate the potential of our approach for assessing changes in gut permeability. Rather, it underscores the need for improved and complementary technologies to assess changes in gut permeability. The fluorescence approach presented here might offer a different representation of the overall stage of the gut barrier, offering advantages in terms of cost-effectiveness, portability and scalability. The next section delves deeper into this by focusing on investigating the potential of the fluorescence sensor to discriminate between different gut permeability conditions using the fluorescence parameters extracted for each studied group.

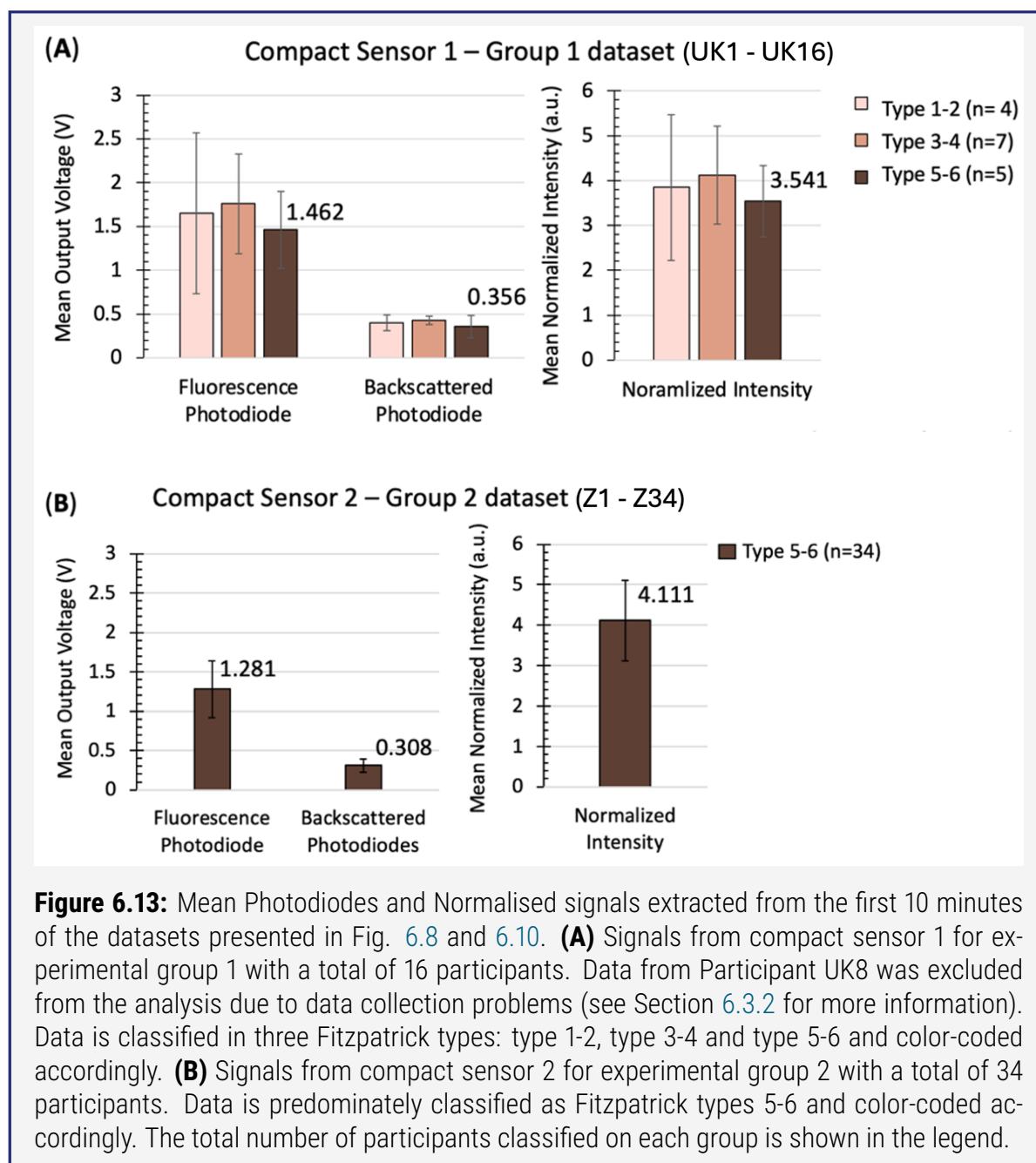
6.3.4 Comparison between Group 1 (Healthy) and Group 2 (Under-nourished) datasets

This section focuses on analysing the differences between the compact sensors (Sensor 1 and Sensor 2) and to characterize the capability of the technology to differentiate between different gut permeability conditions (Group 1 and Group 2). To do so, we firstly analyse the internal responses of the sensors (6.3.4.1. Photodiode signals) under similar experimental conditions. Then, we compare the performance of both sensors under two different conditions by using the fluorescence signals and the parameters extracted from them (6.3.4.2. Fluorescence signals). This will also help us understand the potential of the technology to discriminate between different gut permeability stages. Lastly, we also compare the permeability test parameters (6.3.4.3. Sugar molecules permeability tests – L:M and L:R) as a standard measure of gut permeability for different gut permeability conditions (L:M on Group 1 and L:R on Group 2)

6.3.4.1 Compact sensors - Photodiode signals

Previously, in Section 6.2.2.1, a Calibration factor was introduced to address the electronic variabilities between Sensor 1 and its replica, Sensor 2. The effectiveness of this implementation was validated through an *in vitro* experimental set up. Subsequent sections focused on evaluating the performance of each sensor in monitoring changes in gut permeability, particularly in two *in vivo* experimental conditions: Group 1 and Group 2. In this section, our focus remains on comparing the performance of these compact sensors. This is achieved by analysing the photodiodes' output signals under similar *in vivo* conditions. To do so, the first 10 minutes of the photodiodes' signals (Fluorescence and Backscattered Photodiode) were extracted from the datasets presented in Section 6.3.2 and 6.3.3, from both compact sensors. This ensures that both sensors are analysed

under a similar condition, as fluorescence signals within this time period (10 minutes) are negligible due to the time delay in fluorescein reaching the blood stream following ingestion, so only optical properties from the skin are captured. Fig. 6.13 shows the mean values extracted from the photodiodes and the mean normalized intensities for Sensor 1 (Fig. 6.13A) and Sensor 2 (Fig. 6.13B) for each Fitzpatrick group (Types 1-6). Given that participants in Group 2 are primarily classified into Fitzpatrick type 5-6, comparison between Sensor 1 and Sensor 2 internal photodiodes' response is focused on Fitzpatrick types 5-6 (i.e., skin tone with higher melamine content).



As expected, participants with higher concentration of melanin (Fitzpatrick types 5-6) exhibit lower signals, in both photodiodes, in comparison with the rest of the Fitzpatrick types. This is attributed to the optical properties of the compounds that comprise the skin, specifically to the higher ab-

sorbance property of the melanin pigment. When comparing mean signals from Group 1 and 2, minimal differences were observed between the photodiodes' mean signals for Fitzpatrick types 5-6, with values of 1.462 and 0.356 Volts for Group 1 and 1.281 and 0.308 Volts for Group 2. However, un-paired Student t-tests comparing the individual signals from both groups revealed this difference to be significant ($p\text{-values} < 0.01$ for both cases). This is primarily attributed to the un-balanced number of participants with Fitzpatrick type 5-6 within each group (i.e., Group 1, $n = 5$ vs Group 2, $n = 34$).

In addition, Fitzpatrick classification is highly subjective, and it may not reflect accurately the skin pigmentation (or optical properties) of each participant and thus, estimated Fitzpatrick type on each group can be inaccurate. Nevertheless, comparison of normalized intensity signals from the same Fitzpatrick types 5-6 did not yield significant differences ($p\text{-value} = 0.771$). This indicated that the strategies implemented in the compact sensors, including the use of Backscattered photodiodes, post-processing normalisation and the Calibration factor, address variations between the sensors caused by internal factors (such as electronic configuration) and external factors (such as skin tone).

6.3.4.2 Compact sensors - Fluorescence intensity signals

Previous sections (i.e., 6.3.2 and 6.3.3) have demonstrated the potential of the developed compact sensors on detecting fluorescence permeation from the gut to the bloodstream in different groups of participants. In addition, moderate correlation was found between the fluorescence parameters and the standard permeability tests on different gut permeability conditions (Group 1 in the UK and Group 2 in Zambia), especially between Peak AUC and L:M or L:R ratios. Further analysis, including morphological data, demonstrated non-significant correlation between any of the approaches analysed (including the sugar permeability assays). This supports the idea that biopsies might not represent the entire state of the gut barrier and other approaches, such as the use of fluorescence spectroscopy systems, could provide a bigger picture of the gut permeability state with the advance of portability, immediate results and cost-effectiveness. In this section, we continue analysing the performance of the compact sensors (Sensor 1 and Sensor 2) by comparing the fluorescence intensity signals from Group 1 (with normal gut permeability) with the fluorescence signals from Group 2 (with potential undernutrition). The objective is to assess the capability of the sensors to differentiate between different gut permeability stages, using the parameters extracted from the fluorescence intensity curves.

Fig. 6.14A shows the mean normalized fluorescence vs time curves for Group 1 (*blue*, $n=16$) and Group 2 (*orange*, $n=34$). As expected, both datasets exhibit an increase in fluorescence intensity up to a peak point prior to a decay back towards zero. The time at which peak intensity occurs (in the mean fluorescence signals) varies between groups, with 52 and 65 minutes for Group 1 and 2, respectively. Qualitative analysis of the mean normalized fluorescence intensity curves shows a

clear separation between the groups, with Group 2 exhibiting the highest peak intensity.

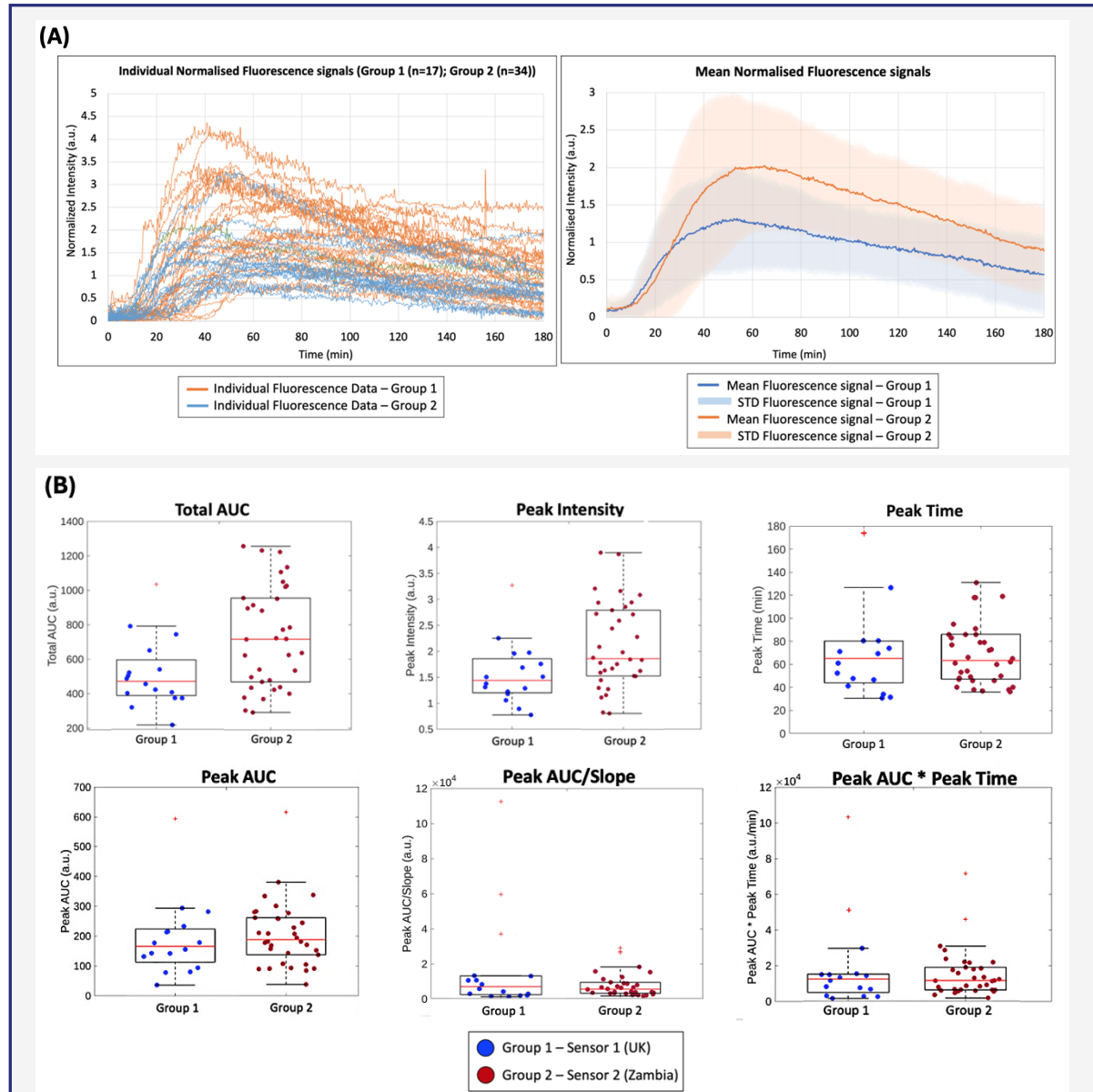


Figure 6.14: (A) Normalised fluorescence signals from each participant (*left*), as well as the mean and STD signals (*right*), for group 1 (blue) and group 2 (orange). The total number of participants in each group (group 1 $n = 16$ and group 2 $n = 34$) is also displayed. Fluorescence signals for each group were collected on different locations using the developed sensors displayed in Figure 6.1 for a total period of 180 minutes. **(B)** Scatter-box plots displaying the parameters extracted from the fluorescence vs time curves – Total AUC, Peak Intensity, Peak Time, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time. Box plots also show median values (red lines), 25th and 75th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for group 1 (UK - blue) and group 2 (Zambia - red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25th percentile or above the 75th percentile by more than 1.5 times the interquartile range.

Fluorescence parameters – Total AUC, Peak AUC, Peak Time, Peak Intensity, Peak AUC/Slope and Peak AUC*Peak Time – were extracted from each individual signal. Scatter-box plots, presented in Fig. 6.14B, show the comparison between each parameter on each group (Group 1 in blue and Group 2 in red). A high degree of overlapping between both groups is observed when comparing the individual values for each participant, especially for Peak Time, Peak AUC and the corrected Peak AUC parameters. This is mainly attributed to the expected inter-patient variability within each group due to changes in gastric emptying rates, diet or the effect of other compounds within the ingested solutions on each group. For example, Group 2 ingested a solution in which Lactulose and Rhamnose, among other molecules, were included. Previous studies have shown that Lactulose, but also stress, exercise, diet and alcohol consumption, for example, can impact the intestinal function, leading to variations in the translocation of molecules, including fluorescein [2, 29, 35, 248]. The effect of these confounding factors in the permeation of fluorescein is yet unknown. However, it is likely that this effect increases the complexity of the absorption kinetics of fluorescein, and any other marker such as Lactulose and Rhamnose, altogether.

A comparison between the mean parameter values - mean Total AUC, mean Peak Intensity, mean Peak Time, mean Peak AUC, mean Peak AUC/Slope and mean Peak AUC*Peak Time, could therefore, minimize the effect of this variability. Table 6.12 shows the mean values of the parameters extracted from the individual fluorescence intensity curves, as well as the *p-values* obtained from the unpaired Student t-test.

Table 6.12: Mean values extracted from the individual values presented in Table 6.4 and 6.6 and shown in Fig. 6.14.

Statistical analysis - Group 1 vs Group 2 (<i>p values</i>)						
	Total AUC	Peak Int	Peak Time	Peak AUC	Peak AUC / Slope	Peak AUC * Peak Time
Group 1	512.804	1.563	74.625	191.625	17601.503	18741.152
Group 2	729.135	2.338	68.501	217.191	7911.191	15209.282
<i>P value</i>	0.0106	0.0078	0.5498	0.4412	0.0726	0.5389

The mean Total AUC and mean Peak intensity observed for Group 2 (729.135 and 2.338 a.u.) were higher than those observed for Group 1 (512.804 and 1.563 a.u.), as shown in Table 6.12 and Fig. 6.14B. This difference was found to be statistically significant using an unpaired Student's T-test (*p-value*= 0.011 and 0.008, respectively). In addition, no significant differences (*p-values* > 0.050) were found when comparing the mean Peak Time, Peak AUC, Peak AUC/Slope and Peak AUC*Peak Time values from Group 1 and Group 2. This is likely due to the similar Peak Time values observed between the groups, together with the wide range of variability within the datasets. The overlapping of the signals was calculated for the first 60 minutes of data collection, resulting in 90.491% and 59.074% of overlapping between Group 1 and 2, respectively. This overlapping further increases the similarity between the two groups (see Table 6.12: Peak AUC and Fig. 6.14B), which is also

supported by the fact that Peak AUC values are calculated using the Peak Time values, which in this case are very similar between the groups.

More importantly, this overlapping effect does not significantly impact the mean Total AUC, as it is reduced to 60.831% and 45.811% for Group 1 and 2, respectively, when the entire signal is considered. This is also true for the mean Peak Intensity, as this parameter relies on the average maximum intensities within each group, calculated using the y-axis component of the signals (with significantly less overlapping).

Considering the significant difference observed between the fluorescence intensity signals from both groups, we used the Peak Intensity parameter to further analyse the datasets, focusing on the different populations within these two groups. To do so, we categorised the participants from both groups taking into account their sex, age and body mass index (BMI). It is important to mention that interpretation of the results is carried out taken into account the limited data available. Fig. 6.15 shows the Peak Intensity values extracted from each participant from Group 1 and 2, classified into the different subgroups. Results from the unpaired Student t-test performed on each subset is presented in Table 6.13. Although limited data is available for females in Group 1 (healthy controls), a significant difference (p value = 0.001) is observed in the male volunteers between Group 1 (healthy) and 2 (undernourished). When analysing the rest of the subgroups, significant differences between Group 1 and 2 is observed in participants older than 35 years old and/or BMI higher than 25, with p values of 0.0132 and 0.0405 respectively.

Table 6.13: Mean values extracted from the subgroups presented in Fig. 6.15. The results from the unpaired student t-test are also included. Significant values are highlighted in bold.

<i>P</i> values from the Pearson's correlation coefficient						
Parameters	Sex		Age		BMI	
	Female	Male	< 35 years	> 35 years	< 25	> 25
Group 1	2.064	1.397	1.615	0.478	1.623	1.501
Group 2	2.332	2.335	2.227	2.605	2.309	2.371
<i>P</i> value	0.524	0.001	0.063	0.013	0.109	0.041

Overall, results presented in this section indicate differences in the gut permeability between Group 1 and Group 2, which further supports the hypothesis that the developed sensors are capable of detecting changes in gut permeability through the permeation of fluorescein from the gut to the bloodstream. These results also suggest that participants from Group 2 exhibits higher gut permeability, in comparison with Group 1, due to the observed increased of fluorescein translocation which could be an indicative of leaky gut. Results from the sex, age and BMI analysis also supported this idea and provided further insights on the capabilities of the developed sensor on assessing changes in gut permeability in different populations. Next section will focus on the analysis and comparison

of standard permeability tests carried out in Group 1 and 2 (i.e., L:M and L:R, respectively) to further understand the differences in gut permeability between the two groups.

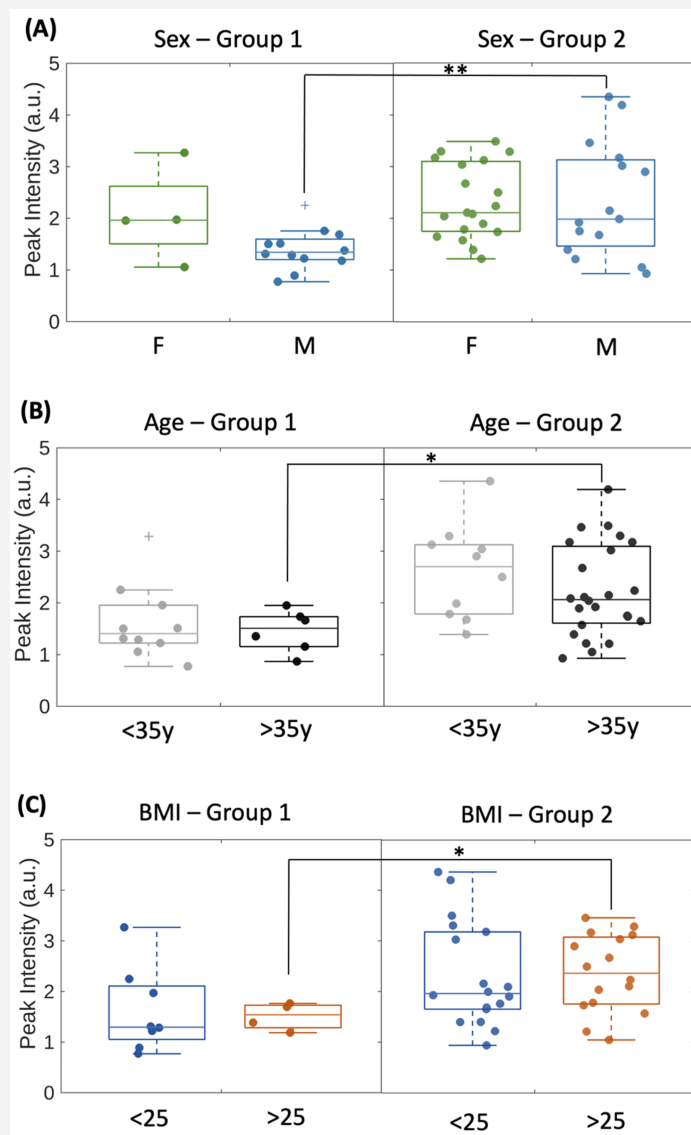


Figure 6.15: Scatter-box plots of the Peak Intensity values from each participant in Group 1 – healthy and Group 2 – undernourished, categorised into three groups: sex (female – F or male – M), age (younger or older than 35 years old) and BMI (threshold of 25). **(A)** Peak Intensity values as a function of sex. **(B)** Peak Intensity values as a function of age, with the threshold established at 35 years old. **(C)** Peak Intensity values as a function of BMI, with a cut value at 25. * - p value < 0.05; ** - p value < 0.001.

6.3.4.3 Sugar molecules permeability tests

As explained previously, all participants from Group 1 and 2 undertook a sugar molecule permeability test. Volunteers from Group 1 undertook the L:M permeability assay no less than a week after the transcutaneous fluorescence experiment. This included a solution of 10 grams of Lactulose and 5 grams of Mannitol. Whereas, volunteers from Group 2 undertook the L:R permeability assay

at the same time as the transcutaneous fluorescence experiment. The solution included 5 grams of Lactulose and 1 gram of Rhamnose, and 200mg of fluorescein. Due to the different permeability assays conducted on each group, comparison between these two datasets focused on the percentage of Lactulose recovered in the urine (%L) of each subject within Group 1 and 2. This is because the use of different sugar molecules, such as Mannitol or Rhamnose, although they have similar molecular weights, they might represent different gut permeability scenarios. For example, Mannitol, is a sugar molecule present naturally in the body and is also present in food and cosmetics. Baseline concentrations of Mannitol can, then, alter the interpretation of the results, specially if no baseline measurements are collected or available. The use of the Lactulose:Rhamnose test has therefore become more popular, as Rhamnose has shown to correlate better with pathological permeability stages [137].

In this case, we firstly compared the percentage of Lactulose, as the molecule common in both groups. Table 6.14 shows the L:R values for each participant within Group 2 that were used. In this case, Group 2 included all the participants from the initial cohort of 80 which %L and L:R ratio was available. This is because, here, we are not considering the fluorescence signals but the L:R test, and therefore, we can include additional participants (from the original 80 participants' cohort). For Group 1, only 14 participants had %L, (see Table 6.5).

Table 6.14: Percentage of Lactulose (%L) and Rhamnose (%R) recovery from participants in group 2 used for the comparison with group 1. In this case, all participants with %L available are included, which means that from a total of 80 participants, $n = 42$ participants are used (this includes data shown in Table 6.7). Data from (%R) is also included to showcase the limitations of the test.

Group 2 (n= 42)											
ID	%L	%R	ID	%L	%R	ID	%L	%R	ID	%L	%R
Z2	0.065	0.253	Z35	0.099	-	Z53	0.442	-	Z68	0.078	0.69
Z3	0.131	-	Z37	0.362	5.958	Z54	0.068	2.893	Z69	0.189	3.765
Z4	1.329	12.069	Z38	-	1.412	Z55	0.031	0.252	Z70	0.093	2.413
Z5	0.065	-	Z39	0.403	4.237	Z57	0.981	-	Z71	0.194	3.289
Z6	0.202	-	Z41	0.353	6.52	Z58	-	2.877	Z72	0.141	2.557
Z7	0.023	0.623	Z43	0.044	1.398	Z59	1.199	12.117	Z73	0.084	1.791
Z11	-	0.248	Z45	0.095	2.141	Z61	0.39	6.624	Z74	0.369	-
Z25	-	1.145	Z46	0.21	3.221	Z62	-	1.237	Z75	0.348	3.347
Z30	-	0.256	Z47	0.063	0.76	Z63	0.092	2.624	Z77	0.329	7.295
Z31	0.095	2.591	Z48	0.114	0.831	Z64	0.033	1.17	Z78	0.158	2.009
Z33	0.509	6.224	Z50	0.166	1.556	Z65	0.069	1.728	Z79	0.574	12.635
Z34	0.107	3.209	Z52	0.947	14.751	Z66	0.123	2.205	Z80	0.015	0.276
										Mean	0.271
										STD	0.315
											3.493
											3.652

The mean %L for Group 1 and 2 were calculated to be 0.182 (range 0.055 to 0.346). and 0.279 (range 0.023 to 1.329), respectively. Fig 6.16 shows the scatter-box plot of the %L for each group.

Contrary to what we saw in the previous sections when using the fluorescence signals, the comparison between %L for Group 1 and Group 2 gave a non-significant difference (p value = 0.353). This could initially be attributed to the disparity in the number of data points available for comparison (i.e., Group 1 with $n = 14$ and Group 2 with $n = 42$). Nevertheless, the high inter-patient variability within each group, together with the differences in the protocols used for the L:M and L:R test, are the potential factors impacting the ability to discern between the groups and the interpretation of the results.

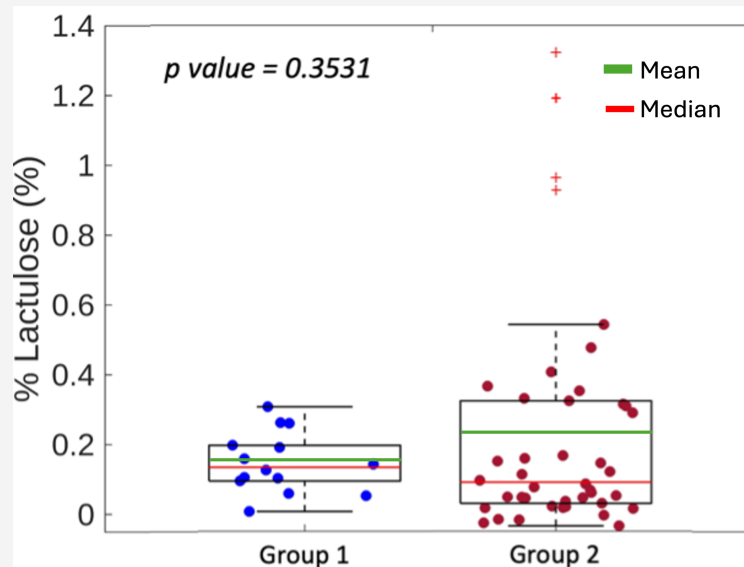


Figure 6.16: Scatter-box plot displaying the individual and mean values of percentage of Lactulose (%L) extracted from group 1 (blue) and group 2 (red). Box plots also show median values (red lines), mean values (green lines), 25th and 75th percentiles (upper and lower bounds of black box), most extreme data points excluding outliers (whiskers), and outliers (red crosses) for group 1 (UK - blue) and group 2 (Zambia - red). Outliers, shown for visualization purposes only, were defined as points that fell below the 25th percentile or above the 75th percentile by more than 1.5 times the interquartile range. Mean values are calculated using all data points, including the outliers.

Considering the differences between the protocols used on both groups, it is also tentative to attribute the lack of significant difference between the groups to the differences in the Lactulose ingested on each group (i.e., 10 grams for Group 1 and 5 grams for Group 2). However, Van Nieuwenhoven et al., for example, showed that differences in the initial dose of Lactulose (range from 1gr to 10gr) within volunteers with normal and induced increased gut permeability did not exhibit significant differences on the percentage of Lactulose recovery, specifically between 5gr and 10gr [134].

On the other hand, we could also speculate that the lack of difference between the groups is due to the effect that high doses of Lactulose have in the gut function. Previous studies, for example, have shown that high doses of Lactulose (for example, range 67 to 134 grams/day or 10 to 30 grams/-

day) could induce an increase in colon transit [248, 249]. In addition, Lactulose is also used to treat colonic constipation, which, in high doses, could induce diarrhoea (i.e., altering the gut permeability) [250]. However, none of the healthy participants reported any abnormal symptoms during the L:M test. In addition, the highest dose of Lactulose was limited to 10 grams, which is lower than the values reported in literature associated with changes in gut motility and permeability.

To further investigate these tests as markers of gut permeability, we also analyse the correlations between the percentage of Lactulose and Mannitol or Rhamnose recovery, as well as the L:M and L:R ratios (see Fig. 6.17). Pearson's correlation coefficient (r) and the resulting p values are shown in the graphs.

As we have previously shown, L:M ratios from Group 1 (mean = 0.010, range 0.003 to 0.019) agreed with values reported in the literature for healthy subjects (mean = 0.0394 ± 0.0235) [130]. Considering this, L:R ratios from Group 2 (mean = 0.079, range 0.037 to 0.259) did fall outside of the range for healthy subjects (mean = 0.0035, range 0.0016 to 0.0080)[134], which suggested an increased in gut permeability of Group 2.

When analysing the data separately, as we can see in the Fig. 6.17, %M and %R exhibit positive correlation with the %L, which suggests that all three sugar molecules offer insights into gut permeability. A weak positive correlation found between the %L and %M was expected, as %M is known to permeate in the majority of GI conditions, including in "healthy" participants. This is the reason why %M is commonly used, together with %L, to calculate the L:M ratio to normalise for differences in gastroemptying rates, slow gut motility, etc. This, therefore, explains the positive correlation found between the %L and the L:M ratio ($r = 0.745$, p value = 0.002)) and support the idea of L:M ratio as a parameter to measure gut permeability.

More interestingly, %R and %L had a considerably high correlation, indicating that both molecules permeated to a similar extent. This, therefore, resulted in a poor correlation between the L:R ratio and the %L ($r = 0.144$, p value > 0.05). In this case, volunteers in Group 2 are potentially undernourished (i.e. increased gut permeability) given the L:R ratios observed and therefore, it is expected that Rhamnose also translocated significantly through the gut barrier. However, in this case, the use of L:R ratio as a marker of permeability might be challenging to directly compare to Group 1 using the L:M ratio.

In addition, upon investigating the L:M and L:R tests simultaneously, we observed baseline levels of Rhamnose in participants where Lactulose and Rhamnose molecules were not provided during the experiment (i.e., participants Z11, Z25 and Z30) due to an issue during the procedure (see Table 6.14). Overall, these observations tentatively suggests that, although the L:R test has become a more popular alternative to the L:M test, this last could be a better indicator of gut permeability. This is only a very tentative observation, as multiple factors, such as participants' clinical history, diet, exercise, urine collection sampling and lack of standardisation between protocols can significantly impact the results and their interpretation.

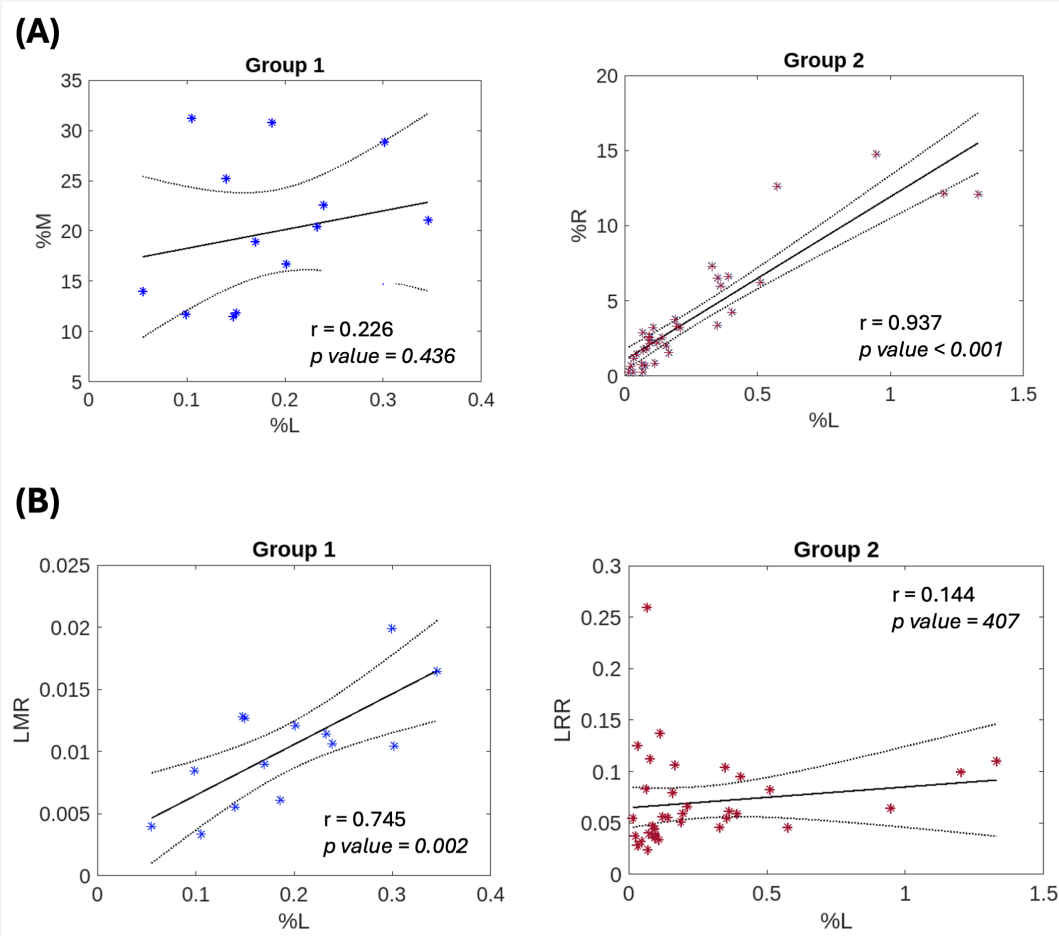


Figure 6.17: Linear regression models applied to percentage Lactulose, Mannitol and Rhamnose presented in Table 6.5 and 6.14. The Pearson's correlation coefficient is represented by the ' r ' value. Its corresponding p-value is also included. **(A)** Correlations between the percentage of Lactulose recovery (%L) and the percentage of Mannitol recovery (%M), for group 1 (healthy controls), and the percentage of Rhamnose recovery (%R), for group 2 (undernourished). **(B)** Correlations between the percentage of Lactulose recovery (%L) and the Lactulose:Mannitol ratio (LMR), for group 1 (healthy controls), and the Lactulose:Rhamnose ratio (LRR), for group 2 (undernourished). In the case of group 2, data includes values from within the entire cohort of participants recruited in Zambia ($n = 80$), with a total of 42 data points. In the case of group 1, total number of data points is 13.

The observations from this sections mainly suggest that relying solely on $L:M$ or $L:R$ ratios might not provide sufficient comprehensive information about gut permeability due to the dynamic interplay between the two sugar molecules (i.e., Lactulose and Rhamnose) nullifying each other's effect. In addition, the use of $L:M$ or $L:R$ ratios have proved to be very individualised among participants and studies. Gan et al., for example, demonstrated the high heterogeneity of $L:M$ ratios reported between different studies and the impact that lack of standardisation has in the interpretation of these results [142].

The research focus should then be to understand what markers are better suited for assessing gut

permeability. As we have seen previously, fluorescein provided a significant difference between the studied participants, while positively correlating with the percentage of Lactulose recovery (%L) when both markers were used simultaneously. This could tentatively point to fluorescein as an effective maker of gut permeability, which also aligns with Ordiz et al. suggesting the use of a single molecule to give a better representation of the gut permeability [125]. However, it is important to consider that interpretation of molecular translocation, including fluorescein, between the studies presented in this thesis and in the literature is significantly limited by the lack of standardisation between tests (i.e., different sugar or fluorescence molecules and concentrations, sample collection times, etc).

Overall, although non-significant difference was found between the %L from Group 1 and 2, the observations presented here showcase the limitation associated with the sugar permeability tests currently used in the clinic, especially when different protocols are used, and support even further the importance of more robust alternatives to assess gut permeability.

6.3.4.4 Advanced analysis for automatic classification of fluorescence datasets

To further demonstrate the potential of the fluorescence signals collected using the developed portable sensor, we analysed the datasets from both groups using a more advanced analysis method. The analysis presented in previous sections focused on implementing the strategies described on Chapter 4, especially the extraction of features from the fluorescence signals – Total AUC, Peak Intensity, Peak Time, Peak AUC, Peak AUC/Slope and Peak AUC * Peak Time - to describe the permeability of fluorescein in different gut barrier conditions. This included the parameters analysed in Section 6.3.2 and 6.3.3 for both of the studied groups – Group 1 from UK and Group 2 from Zambia. We saw that some of these parameters, especially the Peak Intensity, provided significant difference when comparing the two groups, which showcases the potential of the developed fluorescence sensor to assess changes in gut permeability. Another more advance strategy described in Chapter 4, included the calculation of the phasor components from the fluorescence signals and the implementation of a Support Vector Machine (SVM) model to classify the dataset from both groups. Following this approach, a proof-of-concept SVM model was implemented in this chapter to further investigate the potential of this strategy for the classification of fluorescence signals from different permeability conditions. The Real and Imaginary components were extracted in MATLAB, using the following equations:

$$g(w) = \int_0^{180} I(t) * \cos(wt) dx \quad (6.5)$$

$$s(w) = \int_0^{180} I(t) * \sin(wt) dx \quad (6.6)$$

, where $I(t)$ is the fluorescence intensity, t represents the extension of the data collection (180 minutes) and w is the frequency described as: $w=2\pi * \frac{1}{180min}$. Table 6.15 shows the real (Re) and imaginary (Im) components of the fluorescence signals from each participant. Following the machine learning strategy presented in Chapter 4, the Re and Im components extracted previously were implemented into a simplified SVM classifier. The SVM algorithm is widely used for two class classification [225]. Its objective is to find the optimal hyperplane that maximises the separation between the data points within these two groups.

Table 6.15: Phasor components (Real and Imaginary) extracted from the fluorescence signals for each participant in group 1 (UK) and 2 (Zambia). Participant UK8 from group 1 has been excluded from this analysis, due to a problem during data collection (refer to Section 6.3.2). Real - Real number; Imag - Imaginary number.

Group 1			Group 2					
ID	Real	Imag	ID	Real	Imag	ID	Real	Imag
UK1	-13.5243	-145.0829	Z2	-149.51	-16.859	Z20	-197.382	39.345
UK2	-119.4846	55.582	Z3	-61.606	84.145	Z21	-307.395	221.517
UK3	-52.726	-51.7784	Z4	-297.249	44.488	Z22	-235.68	-13.266
UK4	-41.774	76.079	Z5	-121.336	63.866	Z23	-246.157	-137.493
UK5	-187.134	150.455	Z7	-63.945	-48.257	Z24	-211.021	202.217
UK6	-52.321	37.252	Z8	-287.93	65.712	Z25	-190.383	-31.621
UK7	-177.55	52.388	Z9	-137.945	-27.88	Z26	-199.543	156.81
UK9	-41.451	-23.108	Z10	-172.616	-145.067	Z27	-161.991	-40.452
UK10	-115.648	53.162	Z11	-123.347	148.744	Z70	-136.795	-4.705
UK11	-96.927	-39.768	Z12	-183.651	-37.054	Z71	-139.906	-46.457
UK12	-120.623	78.188	Z13	-222.327	82.328	Z72	-105.127	24.432
UK13	-131.631	29.387	Z14	-228.792	-3.703	Z73	-64.402	35.224
UK14	-42.933	108.735	Z15	-120.454	-44.983	Z74	-84.819	155.328
UK15	-62.929	69.382	Z16	-101.715	-87.346	Z75	-113.771	-125.829
UK16	-117.518	182.611	Z17	-193.01	40.721	Z77	-298.747	52.331
UK17	-82.411	15.105	Z18	-264.225	298.169	Z78	-118.995	-29.725
			Z19	-161.953	54.859	Z79	-140.966	26.105
Mean	-94.541	60.759				Mean	-171.903	28.107
STD	49.180	62.380				STD	70.178	100.701

To implement a SVM, the data from Group 1 and Group 2 containing the phasor components is randomised and divided into two groups: training and validation (containing 70% of the data points) and testing (containing 30%). The training and validation data is then implemented into a SVM using the MATLAB function *fitcsvm*. After the validation, the data points within the testing groups are used to assess the final performance of the SVM model. Fig. 6.18 exhibits the decision boundary generated by the SVM after the testing and the data points used for the testing. Fig. 6.18 also displays the confusion matrix generated after the testing.

The resulting SVM model provided an overall accuracy of 86.667%, with an out-of-sample misclassification (or validation error) of 20.134% (obtained from the Leave-one-out cross-validation -

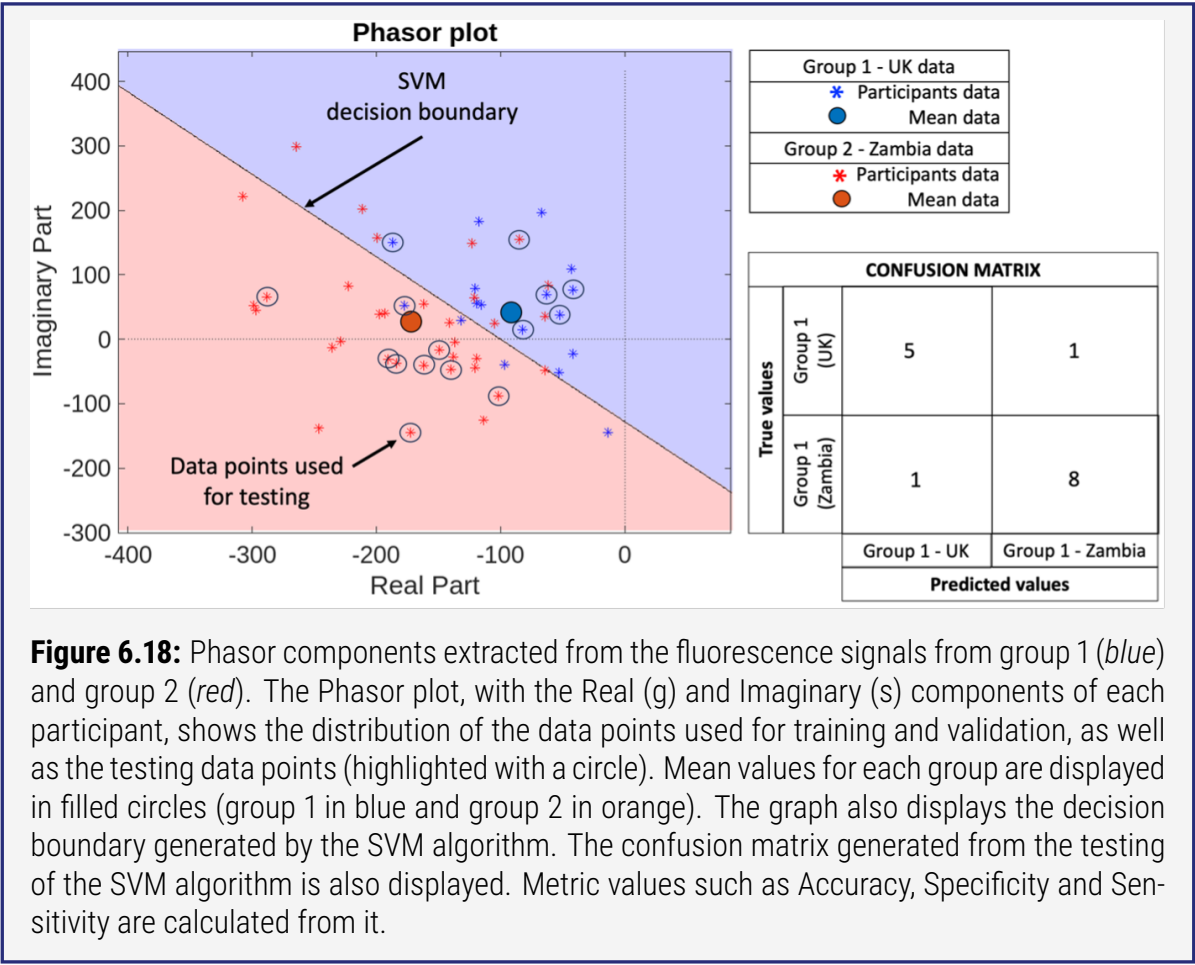


Figure 6.18: Phasor components extracted from the fluorescence signals from group 1 (blue) and group 2 (red). The Phasor plot, with the Real (g) and Imaginary (s) components of each participant, shows the distribution of the data points used for training and validation, as well as the testing data points (highlighted with a circle). Mean values for each group are displayed in filled circles (group 1 in blue and group 2 in orange). The graph also displays the decision boundary generated by the SVM algorithm. The confusion matrix generated from the testing of the SVM algorithm is also displayed. Metric values such as Accuracy, Specificity and Sensitivity are calculated from it.

LOOCV). The sensitivity and specificity of the model, 83.333% and 88.889%, respectively, suggest a good discrimination between Group 1 (UK) and 2 (Zambia) datasets. These results suggest, a priori, that the phasors components could potentially be used as features to automatically discriminate between gut permeability conditions. However, as explained in Chapter 4, it is important to highlight that the results provided by the SVM model do not represent the overall picture of the fluorescein permeability profiles. In practice, the overall performance of a classifiers depends on the size of the dataset, but more importantly, on the level of representation of the distribution of the dataset [251]. With limited data available with high variability, the SVM model is more susceptible to oversee the complexity of the data, which can let to overfitting or biased representations. The use of Machine Learning algorithms in conjunction with additional feature extraction techniques could overcome some of these limitations. The SVM model presented here served, therefore, as a foundational step to promote the development of more complex strategies in the future to capture the full complexity inherent in the fluorescence signals. In addition, it also highlights the advantage that the developed compact sensor provides for the widespread of the technology to increase the number of datapoints collected, otherwise challenging using current clinical approaches such as permeability tests or biopsies. This will help to further understand the gut permeability within different conditions, and how fluorescein permeation correlates with them.

6.4 Conclusion

Nutrient deficiency gives rise to a spectrum of health conditions, including stunted growth, wasting, and underweight disorders, underscoring the pervasive and profound impact of undernutrition on global health. Understanding undernutrition and its relationship with gut health is crucial for developing effective monitoring and diagnostic tools. This chapter has focused on further characterisation of the gut permeability profiles of undernourished volunteers in Zambia by deploying a version of the compact fluorescence sensor presented originally in Chapter 4 (labelled as Sensor 2) and Ref [90]. The original sensor, Sensor 1, was simultaneously deployed in the UK to collect fluorescence intensity signals from healthy volunteers to later compare the signals between both undernourished and healthy groups. The findings also helped to further validate the performance of the compact fluorescence sensors and their potential for assessing changes in gut permeability. The performance of the sensors was, initially, evaluated to establish the differences between both systems to allow for a realistic comparison of datasets, particularly relevant for clinical interpretations. A calibration factor, calculated and validated *in vitro*, was implemented on Sensor 2 for the later comparison between the fluorescence intensity signals from both sensors. In addition, L:M and L:R permeability assay were also performed in the healthy (Group 1) and undernourished (Group 2) volunteers, respectively. Despite the complexity and limitation of the data, as well as the lack of standardisation for the dual sugar permeability tests, the initial *in vivo* results demonstrated significant linear correlation between the fluorescence intensity signals and the L:M and L:R ratios for Group 1 and Group 2, respectively. Fluorescence intensity signals exhibited a range of intensity variations within both groups that were considered normal among individuals and across populations. This variability resulted in a substantial overlapping between the datasets. However, comparison of the fluorescence maximum intensities and the Total AUC values from both groups exhibited significant differences, which showcased the potential of the fluorescence data for assessing changes in gut permeability. Surprisingly, comparison between the percentage of Lactulose recovery (%L) from healthy and undernourished volunteers was found to be non-significant, which was tentatively attributed to the lack of standardisation between the protocols and the presence of human errors during the protocols' implementation. While L:M and L:R dual sugar permeability tests are established as standard clinical approaches for assessing gut permeability, this finding highlighted their limitations and the importance of addressing aspects, such as identifying optimal sugar molecules and establishing standardised procedures for sample collection and storage, including the use of preservatives to prevent substrate degradation.

Further analyses focused on evaluating the correlation between the %L and morphology data available from Group 2, with results exhibiting non-significant correlation. This observation suggested that the parameters extracted from the biopsies might not represent all the changes that occur across the very large surface of the small intestine. In addition, it has also been established that chronic exposure to environmental stressors, such as toxins, infections or malnutrition, can lead

to structural changes in the gut lining [238, 246, 252]. These adaptative responses could confound the assessment of tissues health and the clinical history context of each individual needs to be considered when analysing the biopsy samples.

In conclusion, the results presented in this chapter demonstrated the potential of transcutaneous fluorescence spectroscopy for assessing gut permeability. In addition, the deployment of a portable sensor in a low-income setting, allowed for the further validation of the device and demonstrated the opportunity in enabling the widespread adoption of these devices in clinical settings. The development of portable non-invasive fluorescence systems allowed for the collection of additional data and the further characterization of fluorescence profiles of different gut permeability conditions, with the advantage of portability, cost effective and immediate results. The observations gathered during the in vivo validation also demonstrated the effectiveness of fluorescein, a clinically approved fluorescence contrast agent, on its transcutaneous detection and its selective permeation across the Gastrointestinal tract.

Interestingly, it was also observed that some of the participants from Group 1 (healthy) exhibited high fluorescence intensity signals comparable to those observed in Group 2 (undernourished). While the extent of gut disruption in either group remains uncertain, the presence of these heightened fluorescence signals could suggest two potential scenarios. On one hand, individuals with high fluorescence signals may experience increased permeation of the contrast agent as a result of abnormal gut permeability. Alternatively, it is plausible that fluorescein can permeate the gut barrier even under conditions of normal or healthy gut function, which then will impact the interpretation of the results. Following the last consideration, further work focused on investigating other fluorescence contrast agents that could potentially be used as an alternative of or alongside fluorescein for the improvement of the assessment of changes in gut permeability. More immediate future work will involve the comprehensive analysis of the fluorescence signals together with other relevant factors, such as diet, microbiota and bacterial translocation, to establish their potential correlation.

Chapter 7

In vitro investigation of alternative fluorescence contrast agents in 2D epithelial cell monolayer models

Up to this point the work presented in this thesis has been concerned with the developing of processing algorithms and optical sensors to investigate the potential of transcutaneous fluorescence spectroscopy as a tool to non-invasively assess gut permeability. As mentioned previously, however, when investigating the development of a fluorescence sensor for clinical diagnosis, it is important to consider the specificity of the contrast agent, particularly when focusing on the permeation of this agent in different gut barrier conditions. Fluorescein, as the contrast agent mainly investigated in this thesis, exhibited significant discrimination between different gut permeability stages, proving to be a good candidate for non-invasively assessment of gut permeability. However, it also demonstrated its ability to permeate easily in a broad range of gut barrier conditions established as “normal”, which could impact the reliability of the technology. These findings prompt further investigation into the permeation of fluorescein through the epithelial barrier. Consistent with this idea, we also explore alternative fluorescence contrast agents that could contribute to the improvement of the technology. This chapter presents the results from an in vitro cell monolayer model to investigate the permeation of fluorescein and to compare the efficacy of alternative fluorescence contrast agents in discriminating between gut permeability conditions.

7.1 Introduction

As evidenced in previous chapters, transcutaneous detection of fluorescence signals, after oral ingestion of fluorescein, have proven highly effective in discerning between different states of gut permeability, including those associated with pathological states, such as undernutrition. Upon ingestion, fluorescein, with a molecular weight of 332.306 Da, is absorbed through the gastrointestinal tract. After its permeation from the gut barrier to the bloodstream, fluorescein can be non-invasively detected using transcutaneous fluorescence spectroscopy. The interaction with blood has been reported in the literature showing that fluorescein does bind with plasma proteins [187, 222, 253]. The total volume and rate at which the fluorescein permeates to the bloodstream is indicative of barrier integrity or dysfunction [90, 146, 206, 209, 213]. This characteristic, together with fluorescein molecular weight, its fluorescence profile and its water solubility, allows it to be an effective marker for assessing gut permeability. Results presented in Chapter 6 supported this by demonstrating the potential of fluorescein to discern between healthy and undernutrition participants. However, the high variability found between the healthy volunteers, attributed to normal changes on gut permeability, could potentially suggest that fluorescein easily permeates through the gut barrier. Although, this widespread permeation of fluorescein could provide valuable insights into early abnormal changes of the gut barrier permeability, it could also potentially lead to ambiguity in the interpretation of results. This permeation could be attributed to the relatively small molecular size of fluorescein (332.306 Da) and the mechanisms behind its intestinal permeability. In addition, fluorescein is commonly absorbed in the small intestine, as paracetamol studies have shown [90, 206]. This could limit the range of detection of gut barrier dysfunction, as conditions from other regions could potentially be overshadowed.

Given the heterogeneity of the intestinal tract [254, 255], potential solutions focused on the use of dual contrast agents that could permeate at different locations while allowing for transcutaneous detection. For example, Woting et al. [166], Wang et al. [167] and Ueno et al. [256], showed the potential use of fluorescein isothiocyanate-conjugated (FITC)-dextran to evaluate gut barrier integrity. A previous study conducted in vivo participants showed that, although a 1 gram oral dose of FITC-dextran might not be enough to be transcutaneously detected, urinary recovery of the dye demonstrated its permeation from the gut into the bloodstream [90]. This observation suggests that FITC-dextran, often available in different molecular sizes (4kDa to 2,000 kDa), could potentially be used as a second marker to investigate intestinal permeability at different gut dysfunction stages and at different locations of the Gastrointestinal tract (GI). It is, however, important to mention that both fluorescein and FITC-dextran exhibit similar emission and excitation wavelengths, which could complicate the separation and interpretation of the fluorescence signals from each dye when used simultaneously. Therefore, choosing a different fluorophore with different emission and excitation wavelengths, such as methylene blue, might overcome this problem. Methylene blue (MB), with a molecular size 319.85 Da, is used clinically for the treatment of methemoglobinemia

and as diagnostic tool for sentinel lymph and parathyroid mapping [191–195]. In addition, Borghi-Pangoni et al. [196] showed the use of Methylene blue for the in vitro evaluation of epithelial permeation in animal models. These observations suggest that Methylene Blue could be a potential marker for evaluation of epithelial permeability. However, we cannot ignore the reported adverse reactions associated with Methylene Blue, which include dizziness, headaches or respiratory and vascular problems [197]. Therefore, for the purposes of this chapter, we focus exclusively on the in vitro assessment and comparison of the permeability of fluorescein, FITC-dextran and Methylene Blue.

The investigation of the mechanisms behind the permeation of these contrast agents could provide insights on the benefits that different molecular sizes and fluorescent profiles could have in the assessment of gut permeability. However, the investigation of the paracellular permeability of the previously mentioned contrast agents faces considerable challenges within the complex environment of in vivo experiments and the adverse reactions associated with them [197]. A controlled and simplified model, such as in vitro cell monolayer, could potentially provide some insights into the fundamental process of intestinal permeability for the relevant fluorescence molecules.

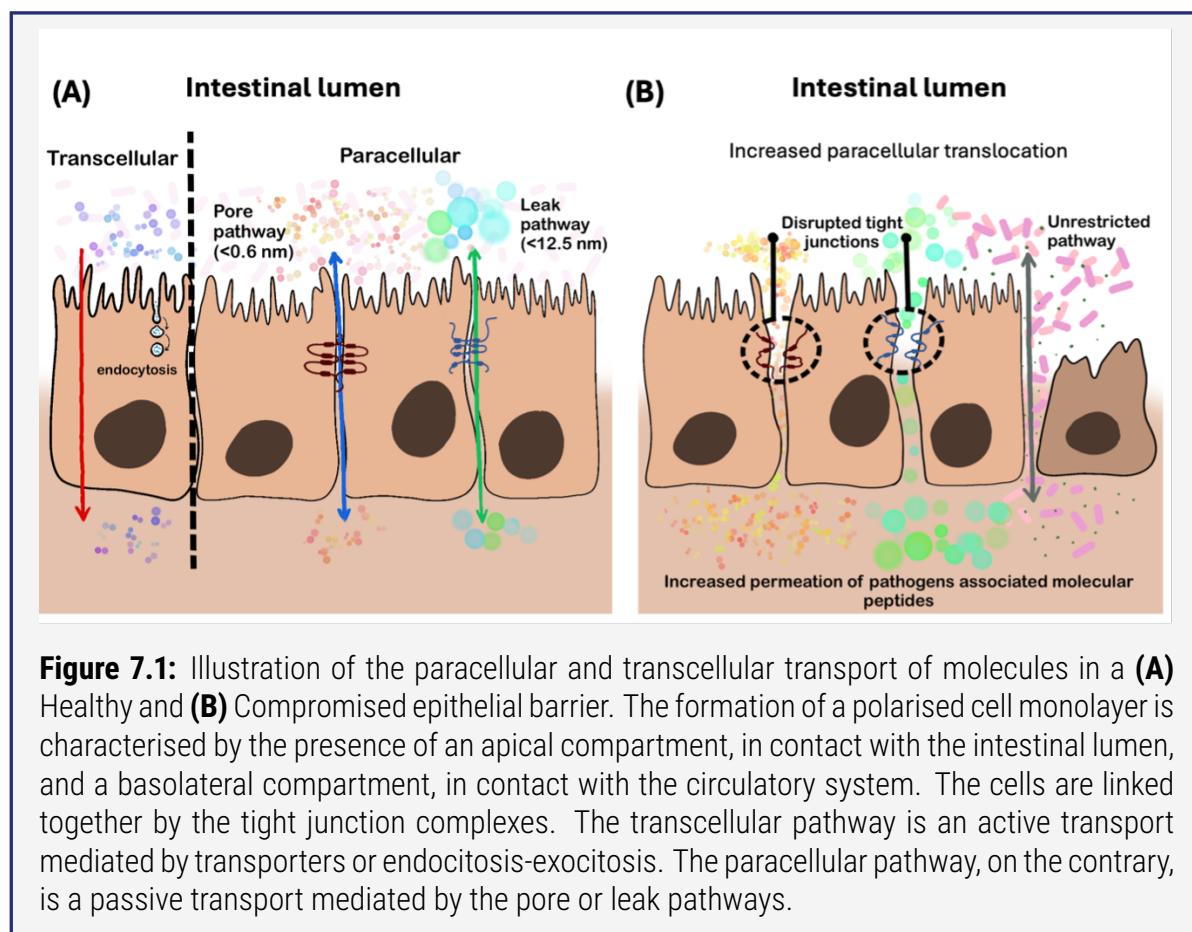
Therefore, in this chapter we present a 2D cell monolayer model to investigate and understand the permeability pathways of different contrast agents, including fluorescein. In addition, the permeability of these contrast agents will also be analysed under different levels of barrier dysfunction. The results obtained from these experiments will, first, help to gain deeper insights into the permeation of the previously mentioned contrast agents across different epithelial barrier conditions. In addition, the use of fluorophores with different molecular sizes and fluorescence spectrum profiles will give us insights about the potential use of these molecules to assess gut permeability in other areas of the GI tract.

7.2 Methods

7.2.1 Gut barrier model in cell monolayer cultures

The gastrointestinal barrier allows the passage of molecules through transepithelial transport, which involves transcellular and paracellular pathways (Fig. 7.1). Transcellular transport facilitates the movement of molecules through cells, and it is mediated by apical and basolateral transmembrane transporters with specific substrate affinities. Contrary, the paracellular pathway, relevant to fluorescein and other fluorescence contrast agents, is less selective and involves movement of molecules across the barrier via charge-selective and/or size-selective pathways [12]. In cases where the gut barrier function is compromised, a third route, known as the unrestricted pathway, originates. In this situation, very large molecules and potentially pathogenic molecules (including bacteria) are able to cross the barrier, contributing to a wide range of intestinal and systemic disorders [25, 26, 31]. Figure 7.1 illustrates the epithelial gut barrier and the permeability pathways:

transcellular in red and paracellular in blue (representing the pore-selective pathway) and green (representing the charge-selective or leak pathway). The third permeability route (unrestricted pathway) is also illustrated in Figure 7.1B. The pore-selective pathway allows the passage of small ions, water and solutes and it is primarily driven by the size of the intercellular spacing. On the other hand, the leak pathway also exhibits selectivity based on the charge of the molecules attempting to pass through.



7.2.1.1 Cell types: epithelial cell model

MDCK (Madin-Darby Canine Kidney) cells and **Caco-2** cells are both commonly used in vitro models for studying epithelial barrier. MDCK cells were derived in 1958 from the kidney of a healthy male Cocker spaniel [257–259]. Caco-2 cells, in the contrary, were derived from human colon adenocarcinoma [260, 261]. Despite their differences in origin, both cells' lines exhibit many of the characteristics of intestinal/renal epithelial cells, including the formation of tight junctions and the formation of polarized monolayers. The polar organisation is characteristic by the formation of both apical and basolateral membrane domains or compartments [262]. Caco-2 cells are directly derived from human tissue and therefore, they better reproduce the human physiology and pharmacokinetics. However, their complexity is also transferred into their requirements for culture and maintenance, as they have a slower growth rate, and exhibit higher variability between passages

compared to MDCK cells [263, 264]. Both cell lines have been extensively used in permeability studies and are a well-established model for drug absorption and epithelial transport [265–267]. However, for the scope of this research, MDCK cells were used on the cell monolayers models presented in this chapter. This is due to their rapid growth and the ease of culturing and handling. MDCK cells form polar monolayers (with apical and basolateral compartments, as illustrated in Fig. 7.1) with well-defined tight junctions that allows the translocations of molecules. In this chapter we will be focused on the translocation of molecules through the paracellular pathway, as this is the mechanism of transport investigated by clinical approaches, such as Lactulose:Mannitol tests, and it is relevant to a wide range of conditions, including malnutrition [29, 36, 86, 125].

7.2.2 Disruption of tight junctions

As mentioned in the previous section (Section 7.2.1), when the polar configuration of the epithelial barrier is damaged, an unrestricted pathway is created. This is referred as gut dysfunction or “leaky gut”. Depending on the severity of the lesion, allergens, pathogens and bacteria can move freely from the apical compartment to the basolateral, causing inflammatory responses and aggravating other pathological conditions [1, 2, 25, 34]. The technique presented in previous chapters focused on the use of transcutaneous fluorescence spectroscopy to assess gut permeability to improve the diagnosis and treatment of conditions associated with “leaky gut”. To understand the impact that “leaky gut” has on the permeation of the fluorescence agents, we artificially induced damage to the cell monolayer models by using ethanol and Ethylene glycol tetraacetic acid (EGTA) as our tight junction disruptors.

Previous studies in conventional two-dimensional (2D) cell monolayers [268, 269], have shown that exposure to ethanol at concentrations $\leq 10\%$ v/v or 10–40 mM lead to disruption of the epithelial tight junctions and thereby increase paracellular permeability [236, 270]. Other studies also shown that EGTA (Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid), a specific calcium chelator, has dramatic effects on the paracellular permeability and transepithelial electrical resistance (TEER) in Caco-2 cells [271–273]. EGTA is a calcium chelator commonly used to disrupt calcium-dependent cell adhesion and tight junction integrity in epithelial cell monolayers such as Caco-2 [274], demonstrating to be an efficient gut barrier disruptor. Given the effectiveness of ethanol and EGTA to affect the integrity of the cell monolayers, we will also investigate different levels of gut barrier damage. This will help to understand more deeply the impact that barrier disfunction has on the contrast agent's permeability. To do so, we titrated the ethanol and EGTA into different solutions with specific concentrations. Section 7.2.4.2, describes the experiments carried out to calculate the concentrations of ethanol and EGTA needed to correlated with severe, moderate, mild and none gut damage.

7.2.3 Fluorescence contrast agents

As mentioned previously, this chapter focuses on investigating the permeability of different fluorescence contrast agents, including fluorescein, on 2D cell monolayer models. The contrast agents included in the experiments are described in Table 7.1, including the molecular weight, quantum yield number and fluorescence profiles. Fluorescein is the contrast agent used on the experiment reported in previous chapters, as it was effectively detected transcutaneously and demonstrated the capability to significantly discern between different gut permeability conditions. Based on the supplier information (Sigma Aldrich - (CAS-number: 518-47-8), fluorescein has a molecular weight of 376.270 Da with an emission and excitation wavelengths of 460 and 515 nm, respectively. In the case of Methylene, it has a similar molecular weight as fluorescein (i.e., 319.850 Da), but with longer excitation and emission wavelengths of 665 and 686 nm, respectively [191, 275, 276]. FITC-dextran, in the contrary, can be presented in multiple molecular weights (4,000 kDa in our case), making it the largest contrast agent used in these experiments. As for the fluorescence profile, FITC-dextran is a marker containing fluorescein isothiocyanate (FITC; a derivate of fluorescein) as the fluorophore and therefore, has an emission and excitation wavelengths similar to fluorescein (490 nm and 520 nm, respectively) [Data from the supplier Sigma Aldrich - (CAS-number: 60842-46-8)].

Table 7.1: Chemical and optical characteristics of the fluorescence agents used in the cell monolayer experiment. This information is based on the manufacturer and it might vary slightly from the literature.

Fluorophore	Molecular weight (Da)	Quantum yield	Fluorescence profiles
Fluorescein sodium salt	376.270	0.910	Excitation: 460 nm Emission: 515 nm
FITC-dextran	≈4,000k	0.920	Excitation: 490 nm Emission: 520 nm
Methylene Blue	319.850	0.520	Excitation: 665 nm Emission: 686 nm

7.2.4 Experimental procedures

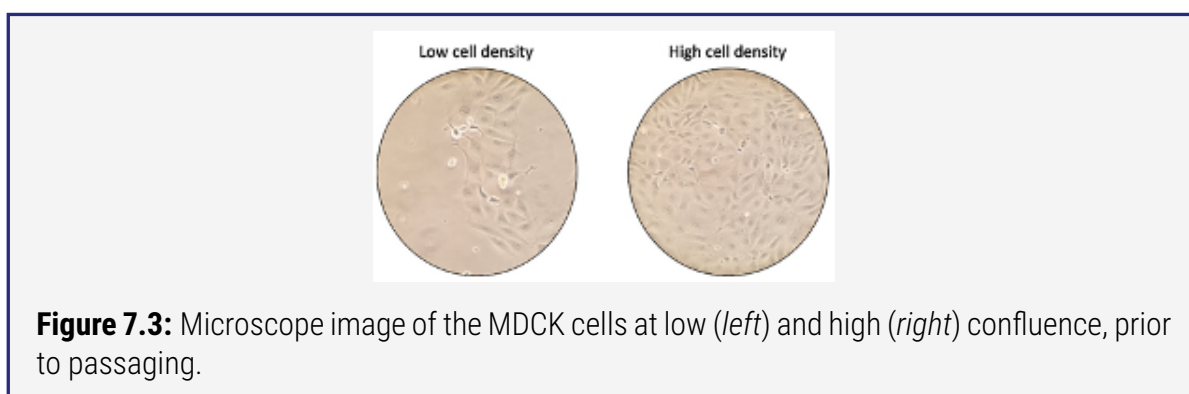
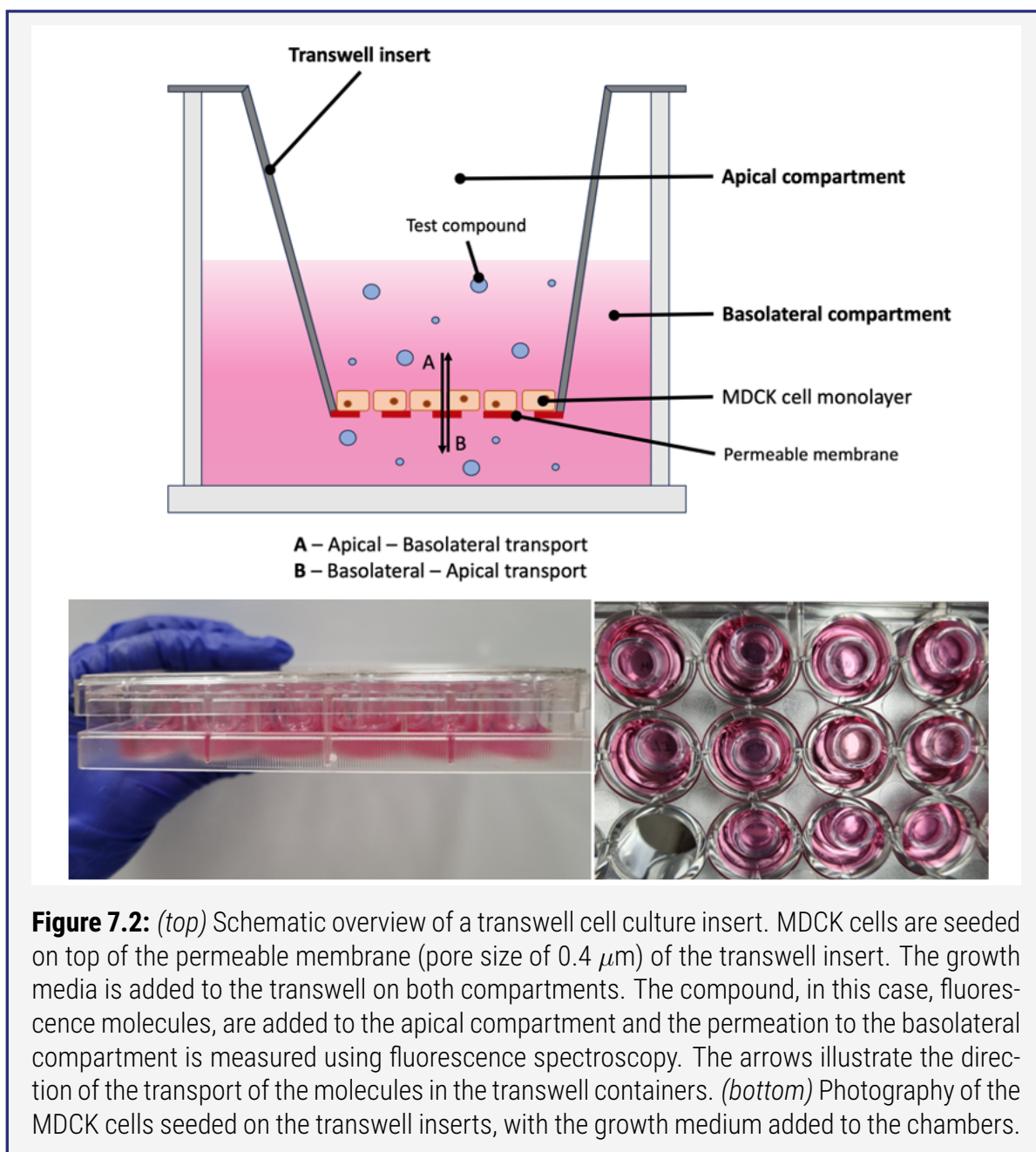
This section provides a description of the experimental procedures conducted to investigate the different fluorophores' permeations. To do so, this section starts by introducing the methodology implemented to create the 2D cell monolayer models used for the experiments (section 7.2.4.1). The section continues describing the different approaches and concentrations used to induced multiple stages of epithelial barrier disruption (section 7.2.4.2). Following this, section 7.2.4.3 introduces the different concentrations of fluorophores used for the experiments, taking into account

their fluorescence properties. A control experiment is also described in section 7.2.4.4 to establish the baseline levels of permeation of the contrast agents. Finally, section 7.2.4.5 provides information about the experimental work carried out to investigate the permeation of each fluorophore under different conditions of barrier dysfunction. The step-by-step protocols used for the various experimental stages are provided in section 7.2.5.

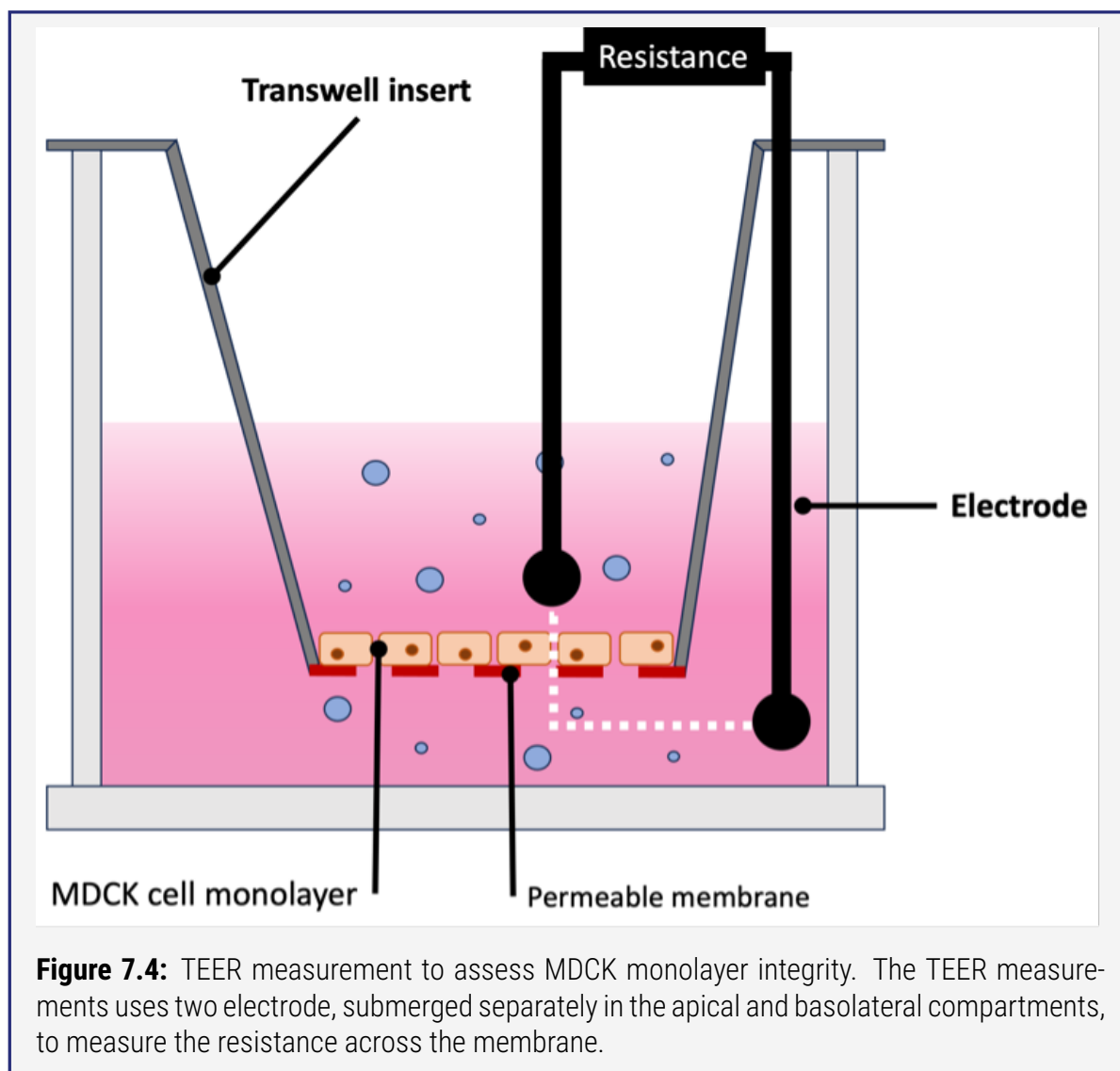
7.2.4.1 Cell monolayer culture

As explained in Section 7.2.1.1, MDCK cell line is used for the 2D cell monolayer models due to its relatively simple culture requirements and their rapid growth (reaching 80% confluence in approximately 2 days). To recreate a 2D epithelial barrier, MDCK cells are cultured on permeable filter supports, i.e., transwells. In this case, we used 24-transwell plates (Corning, Falcon®, REF: 353504), with a PET 0.4 μm pore-size inserts (Corning, Falcon®, REF: 353095). As explained previously, MDCK cells form an apical (A) and basolateral (B) compartment. The apical compartment reproduces the side of the barrier facing the intestinal lumen, whereas the basolateral compartment recreates the side facing the bloodstream (Fig. 7.2). This conformation provides a simplified model of a human gut barrier and therefore, allows for investigation of different permeability pathways on different conditions.

The following method describes how the MDCK cells were maintained and seeded into transwell plates. Medium, containing 500 ml of DMEM (Thermo Fisher Scientific, Gibco™, REF: 21969035), 10% of FBS (Thermo Fisher Scientific, Gibco™, REF: A5256701), 1% of Glutamax (Thermo Fisher Scientific, Gibco™, REF: 35050061) and 1% of Pen/Strep (Thermo Fisher Scientific, Gibco™, REF: 15140122), was prepared in advance and stored in the fridge. MDCK cells were provided by the QMUL-Blizard institute in frozen aliquots. To get them ready for the experiments, the cells were thawed and, initially, seeded in a T25 flask (see Section 7.2.5: Protocol 1 and 2). After approximately 80% confluence was reached, cells were split 1:10 and seeded again on a T25. After a couple of days and before starting the experiments, cells were again split 1:10 and seeded to, this time to a T25 and a T75 flask. The T25 flask was used to seed the cells in the transwell plates, whereas the T75 flask, with higher surface area, was used to maintain the cell line, so fresh cells were always available to seed on new transwell plates if needed. Because of the rapid growth of the MDCK cell line, cells were constantly split 1:10 and passaged every 2-3 days (depending on the level of confluence) to a new T75 flask, with the medium replaced each time. MDCK cells from the T25 flask were seeded in 24-transwell plates with PET permeable inserts (0.4 μm pore size) and used 1 day after plating, when approximately 80% confluence was reached (Fig. 7.3). Cells were seeded in the transwell plate using Section 7.2.5: Protocol 2, at a cell density of 4×10^5 cells/ml.



Prior to use, MDCK cell monolayers were assessed using an Epithelial Volt-Ohm Meter 3 (EVOM3 - World Precision Instruments) to measure the transepithelial electrical resistance (TEER). TEER measurements are used to assess the integrity of epithelial cell monolayer. It involves the measurement of the electrical resistance across a cell monolayer, providing an indirect measure of the tightness of cell-cell junctions. Fig. 7.4 illustrates the process. Prior to TEER measurements, the electrode from the EVOM3 was submerged in medium for approximately 30 minutes to calibrate the meter. TEER measurements were collected following the protocol described in Section 7.2.5: Protocol 4 and analysed in Excel.



7.2.4.2 Disruptor optimisation

The permeation of molecules through the paracellular pathway depended primarily on the integrity of the tight junctions. In this chapter, we aim to investigate the permeability of different fluorescence dyes on different epithelial barrier stages. As explained in Section 7.2.2, Ethanol and EGTA have proved to be efficient tight junction disruptors [269–272] In this case, Ethanol and EGTA were

used, at different concentrations, to simulate different stages of epithelial barrier damage. The experiment carried out in this section helped to decide the specific disruptor concentrations used later in the permeability assay.

The initial concentrations of ethanol and EGTA (labelled as maximum concentrations) were obtained from the literature ([268, 269, 277, 278]) and from previous protocols followed within the laboratory. Table 7.2 illustrates the concentrations used on each case. The process involved the initial preparation of the most concentrated solution to be used. The rest of the concentrations were then obtained by 1:2 titrations. Fig. 7.5 illustrates the process followed to obtain the EGTA solutions. A similar process was followed for the Ethanol. However, to prevent ethanol from evaporating, the ethanol solutions were made fresh every time they needed to be used.

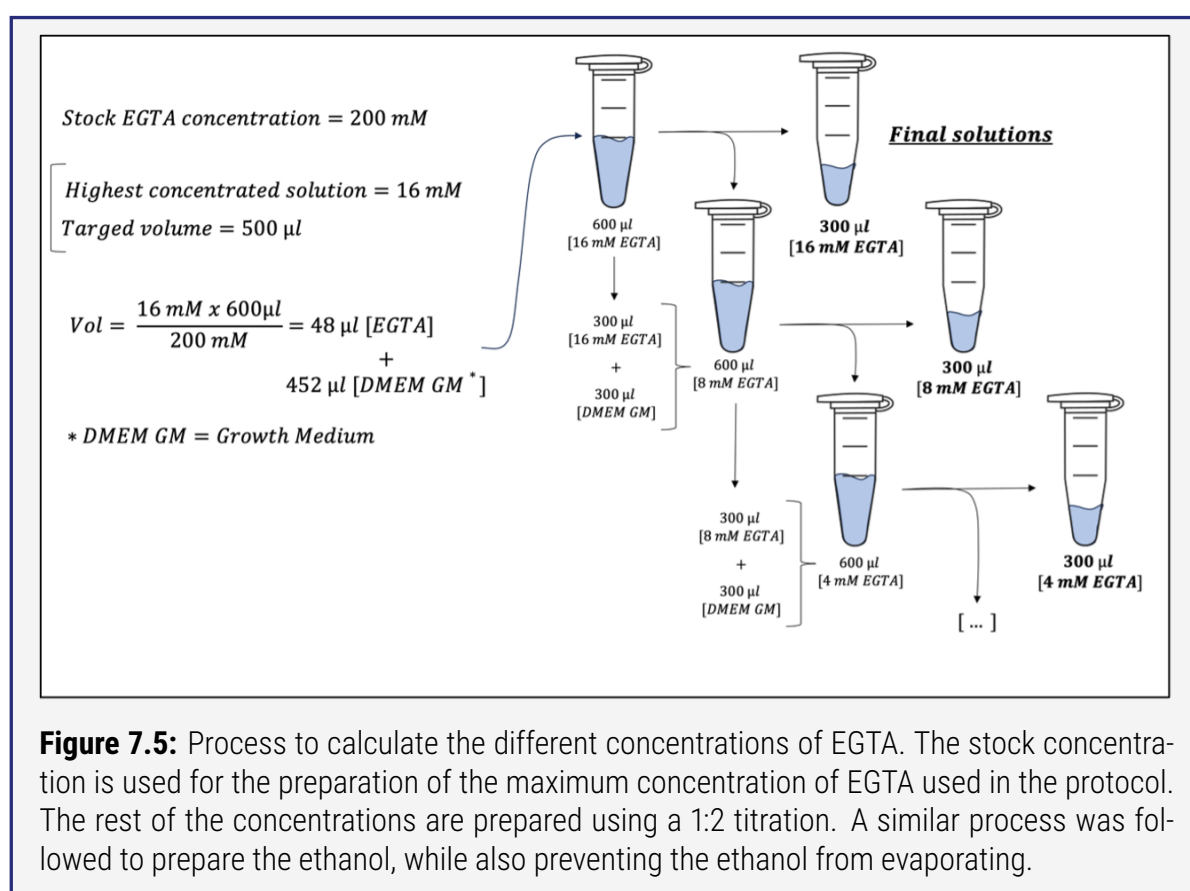


Figure 7.5: Process to calculate the different concentrations of EGTA. The stock concentration is used for the preparation of the maximum concentration of EGTA used in the protocol. The rest of the concentrations are prepared using a 1:2 titration. A similar process was followed to prepare the ethanol, while also preventing the ethanol from evaporating.

MDCK cells used for this experiment were seeded on the transwell plates 1 day in advance. Plates were labelled as shown in Fig. 7.6. Two wells, one with and other without cells, were left as controls. On the day of the experiment, TEER measurements were collected, and data was stored as T0 (t = 0 hours). Then, the medium from the basolateral chamber, firstly, and apical chamber, secondly, were carefully aspirated. In the apical chamber, medium containing the disruptor ethanol or EGTA was added, following the concentrations labelled in the plates, as shown in Fig. 7.6. Basolateral chambers were then filled with fresh medium and cells were placed in the incubator. TEER measurements were collected for a total of 3 hours with measurements at T0 (t=0 hours), 1.5 hours and 3 hours.

Table 7.2: Ethanol and EGTA solutions and the different concentrations used to disrupt the epithelial barrier at different levels. Concentrations were made following the process illustrated in Fig. 7.5. Relative volumes of Ethanol and EGTA are also provided.

	Concentrations							
Ethanol	0 μ l/ml (0%)	5 μ l/ml (0.5%)	10 μ l/ml (1%)	20 μ l/ml (2%)	30 μ l/ml (3%)	40 μ l/ml (4%)	50 μ l/ml (5%)	60 μ l/ml (6%)
EGTA	0 μ l/ml (0 mM)	1.25 μ l/ml (0.25 mM)	2.5 μ l/ml (0.5 mM)	5 μ l/ml (1 mM)	10 μ l/ml (2 mM)	20 μ l/ml (4 mM)	40 μ l/ml (8 mM)	80 μ l/ml (16 mM)

Data was analysed in excel to determine the impact that each disruptor and concentration had on the integrity of the barrier. Two replicates of the experiments (i.e., two plates as the ones showed in Fig.7.6) were performed.

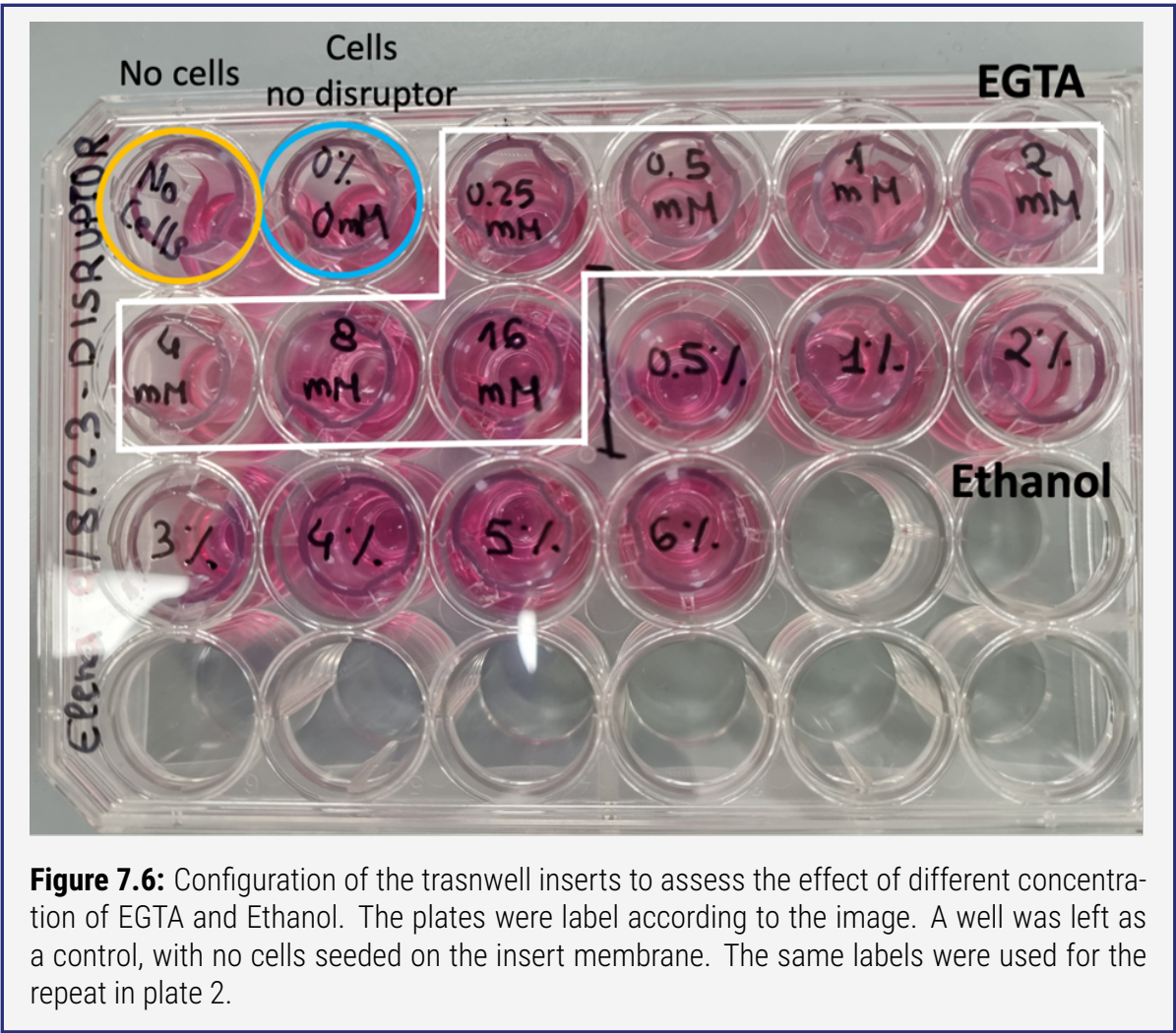


Figure 7.6: Configuration of the trasnwell inserts to assess the effect of different concentra-
tion of EGTA and Ethanol. The plates were label according to the image. A well was left as
a control, with no cells seeded on the insert membrane. The same labels were used for the
repeat in plate 2.

7.2.4.3 Dye optimisation

We firstly focus on investigating the permeability of different fluorescence dyes under normal (no disrupted) conditions. Additionally, we also analyse the fluorescence intensities at different concentrations to investigate other aspects of the contrast agents that can later impact the interpretation of the results. This includes for example the self-quenching effect of the fluorescence dyes, as well as the saturation limit of the spectrometer DARIOstar (from BMG Labtech) [279, 280].

The different fluorescence dyes investigated in this chapter have different quantum yield numbers, as shown in Table 7.1. For the comparison of their permeability, the maximum concentrations were, therefore, extracted taking into account the differences in their quantum yield numbers. This is to ensure that any variations observed when comparing their fluorescence intensity signals are primarily attributed to differences in permeability and not to the differences in their brightness (i.e., efficiency to convert absorbed photons into fluorescence emission).

The maximum concentration of FITC-dextran was initially established at 100 µg/ml, which generated readable fluorescence intensities and was routinely used in the laboratory protocols at Blizzard Institute. In addition, FITC-dextran assays are well-established and standardised in research making it useful as a control for our fluorescence intensity readings [281–284]. Because FITC-dextran and fluorescein share similar fluorescence quantum yields (0.92 and 0.91, respectively), the maximum concentration of fluorescein was primarily determined by the ratio of FITC to Dextran. This ratio of 0.004 mol of FITC:1 mol of dextran, as provided by the manufacturer, resulted on a fluorescein maximum concentration of 0.835 µg/ml. This was calculated as follows:

1. Calculate how many *mols* of FITC-dextran are in 100 µg of FITC-dextran

$$\begin{aligned}\text{FITC-dextran (molecular weight)} &= 4000 \frac{\text{g}}{\text{mol}} \text{ (quoted by the manufacturer)} \\ 100 \mu\text{g FITC-dextran} &= 1 * 10^{-4} \text{ g} \\ \text{mols of FITC-dextran} &= \frac{1 * 10^{-4} \text{ g}}{4000 \frac{\text{g}}{\text{mol}}} = 2.5 * 10^{-8} \text{ mols}\end{aligned}$$

$$\mathbf{A} = 2.5 * 10^{-8} \text{ mols FITC-dextran in } 100 \mu\text{g} \quad (7.1)$$

2. Calculate how many *mols* of Dextran (glucose) are in **A**

- Calculate how many *mols* of glucose are in 1 *mol* of FITC-dextran

$$\begin{aligned}\text{Glucose (molecular weight)} &= 180.156 \frac{\text{g}}{\text{mol}} \\ \text{mols of glucose} &= \frac{4000 \text{g/mol}}{180.156 \frac{\text{g}}{\text{mol}}} * 1 \text{ mol} = 22.203 \text{ mols}\end{aligned}$$

$$\mathbf{B} = 22.203 \text{ mols glucose in } 1 \text{ mol FITC-dextran} \quad (7.2)$$

- Calculate how many *mols* of glucose are in **A** based on **B**

$$\text{mols of glucose} = A \times B = 2.5 \times 10^{-8} \text{ mols} \times 22.203 \text{ mols} = 55.507 \times 10^{-8}$$

$$\mathbf{C} = 55.507 \times 10^{-8} \text{ mols glucose in A} \quad (7.3)$$

3. Calculate how many *mols* of Fluorescein (or FITC) are in **A** based on **C** and the ratio between FITC and dextran

Ratio FITC:Dextran = 0.004:1 (given by the supplier)

$$\text{mols of FITC} = 0.004 \times C = 0.004 \times 55.507 \times 10^{-8} = 2.220 \times 10^{-9} \text{ mols}$$

$$\mathbf{D} = 2.220 \times 10^{-9} \text{ mols FITC in A} \quad (7.4)$$

4. Calculate how many grams of FITC are **D**

$$\text{FITC (molecular weight)} = 376.270 \frac{\text{g}}{\text{mol}}$$

$$\text{grams of FITC in 100 } \mu\text{g of FITC-dextran} = 0.835 \mu\text{g} \quad (7.5)$$

In the case of Methylene blue, it presents a quantum yield number of 0.52. In this case, its maximum concentration was calculated using a correction factor of 1.75 to compensate for the difference in quantum yield with respect to fluorescein. The maximum concentration of Methylene Blue was calculated as follows:

- Correction Factor = 1.75

$$\text{Methylene Blue maximum concentration} = 1.75 \times 0.835 \mu \frac{\text{g}}{\text{ml}} = 1.461 \frac{\mu\text{g}}{\text{ml}} \quad (7.6)$$

Once these maximum concentrations were calculated, we used the recipes in Section 7.2.5: Protocol 4, to prepare the solutions. A 1:2 titration was used to calculate the rest of the concentrations, using medium as solvent. The original concentrations of the fluorescence dyes, labelled as stock solutions or 100%, and the respective titrations are shown in Table 7.3.

For the experiment, MDCK cells were seeded on the transwell plates 1 day in advance. Plates were labelled as shown in Fig. 7.7 (right), with the percentages representing the different concentrations of fluorescence dyes. Three wells were left as controls for the dyes (with cells, but no fluorescence dyes) and one well was left as control for cells (no cells and no dyes).

On the day of the experiment, TEER measurements were collected to ensure the integrity of the barrier and data was stored as T0. Then, the medium from the basolateral chamber were carefully

Table 7.3: Fluorescence concentrations and the different titrations for the contrast agents: fluorescein, FITC-dextran and Methylene Blue. Concentrations were prepared following Section 7.2.5: Protocol 4.

Dye	Control	6.25%	12.5%	25%	50%	100%
FITC-dextran	0 $\mu\text{g/ml}$	6.25 $\mu\text{g/ml}$	12.50 $\mu\text{g/ml}$	25.00 $\mu\text{g/ml}$	50.00 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
Fluorescein	0 $\mu\text{g/ml}$	0.052 $\mu\text{g/ml}$	0.104 $\mu\text{g/ml}$	0.209 $\mu\text{g/ml}$	0.417 $\mu\text{g/ml}$	0.835 $\mu\text{g/ml}$
Methylene Blue	0 $\mu\text{g/ml}$	0.091 $\mu\text{g/ml}$	0.183 $\mu\text{g/ml}$	0.365 $\mu\text{g/ml}$	0.731 $\mu\text{g/ml}$	1.461 $\mu\text{g/ml}$

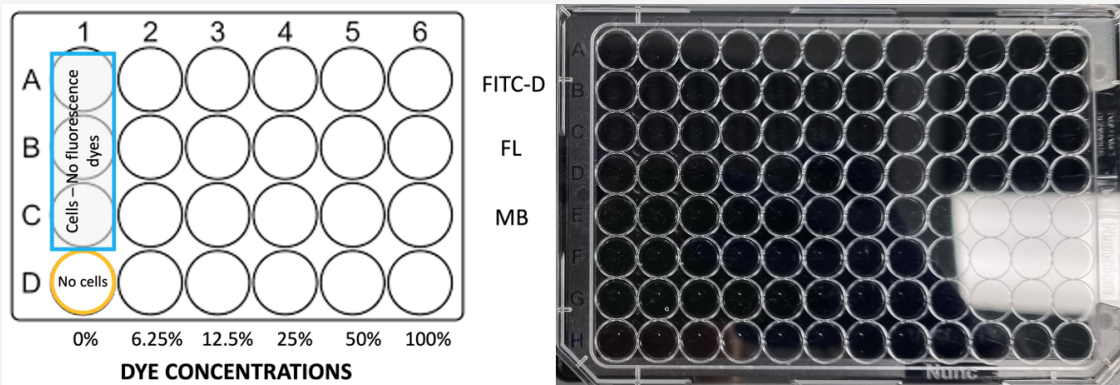


Figure 7.7: (left) Labelling of the plates used for the assessment of the permeation of the fluorescence dyes - FITC-D: FITC-dextran; FL: Fluorescein; MB: Methylene Blue - at different concentrations (0% to 100%). (right) Dark plate used to measure the fluorescence intensity of the dyes, after the experiments. Apical and basolateral samples were added to the dark plate, to measure the fluorescence intensity remaining after 3 hours in both compartments.

aspirated, followed by the volume from the apical compartment. In the apical chamber, medium containing the fluorescence dye was added, with the concentrations specified in the plate, as shown in Fig. 7.7. Basolateral chambers were then filled with PBS and cells were placed in the incubator for 3 hours. TEER measurements were collected for a total of 3 hours at intervals of 1 hour each. At the end of the 3 hours period, 100 μl from the apical and basolateral chambers were transferred to a dark plate, with 2 repeats per chamber and well. Fluorescence intensity readings were collected using the DARIOstar plate reader (from BMG Labtech, Fig. 7.7 (right)) and data was analysed in Excel to determine the permeability of each fluorescence dye and concentration on un-disrupted cell monolayers. As illustrated in Fig. 7.1, the diffusion of the fluorescence dyes in the 2D cell monolayers occurs from the apical to the basolateral, but also from the basolateral to the apical. It is expected that the diffusion of the fluorescence molecules continue until an equilibrium is reached (i.e. concentrations at the apical and basolateral compartments are balanced). Therefore, the permeability of the fluorescence dyes is assessed using the relative fluorescence intensity signals at the basolateral compartment (F_B), with respect to the apical compartment, the initial concentration and the time at which the measurements were done. This is calculated as follows:

$$F_B' = \frac{F_B(t = 3h)}{F_A(t = 3h) + 5 * F_B} * F_0 \quad (7.7)$$

, where $F_A(t=3h)$ and $F_B(t=3h)$ are the average fluorescence intensity signals measured at the apical and basolateral compartments (at $t = 3$ hours), respectively. F_0 is the initial fluorescence signal for each dye and concentration. In addition, the differences in the volumes within the apical ($200 \mu\text{l}$) and basolateral (1 ml) compartment is also taken into account by implementing a factor of 5 within the equation. Fluorescence signals from the Growth medium and PBS are also extracted to the F_0 , F_A and F_B values in advance. Since the concentrations of fluorescence dyes is expected to reach an equilibrium, measurements are collected at $t = 3$ hours, to ensure that values in the basolateral compartment have not reached the equilibrium state. As before, at least 2 replicates of the experiments were performed.

7.2.4.4 Transwell insert - control permeability

As explained in previous sections, paracellular permeability of molecules is commonly influenced by the size of the substrate to be translocated. In our model, the permeation is mainly attributed to the integrity of the tight junction of the epithelial barrier. However, the transwell inserts, with $0.4 \mu\text{m}$ pore size, could potentially impact the translocation of the molecules, especially those fluorescence dyes with larger molecular size, such as FITC-dextran. To investigate this, we followed a similar protocol as the one used on Section 7.2.4.3, in which the permeation of the fluorescence dyes from the apical to the basolateral compartment is measured using fluorescence spectroscopy. Two replicates of the experiments were performed. However, MDCK cells were not used in this case, as only the impact of the membranes is investigated.

7.2.4.5 Permeability assay

This last section describes the protocol followed to investigate the permeation of different fluorescence dyes – Fluorescein, FITC-dextran and Methylene blue – on 2D MDCK monolayers with different permeability conditions, as induced by different concentrations of the disruptors – Ethanol and EGTA. These experiments include the results from previous sections, such as the optimised concentrations for the fluorescence dyes and the disruptors.

In advance to the experiments, MDCK cells were seeded in 4 different transwell plates. A total of 24 wells per fluorescence dye and 13 additional wells for control were seeded, as illustrated in Fig 7.8. On the day of the experiment, TEER measurements were collected from all the wells to ensure the integrity of the barrier and data was stored as T0. Then, the medium from the basolateral chamber were carefully aspirated, followed by the volume from the apical compartment. In the apical chamber, medium containing the fluorescence dye and the disruptor were added, with the relevant concentrations specified in the plate, as shown in Fig. 7.8. Basolateral chambers were then filled with PBS and cells were placed in the incubator for 3 hours. TEER measurements were collected

for a total of 3 hours at intervals of 1 hour each, to evaluate the level of barrier damage induced by the disruptors. At the end of the 3 hours period, 100 μ l from the apical and basolateral chambers were transferred to a dark plate, with 2 samples per compartment and transwell. Fluorescence intensity readings were determined using the DARIOstar plate reader (from BMG Labtech, depicted in Fig. 7.6 (left)) and data was analysed in excel to determine the permeation of each fluorescence dye and concentration at every level of induced damage. As before, at least 2 replicates of the experiments were performed.

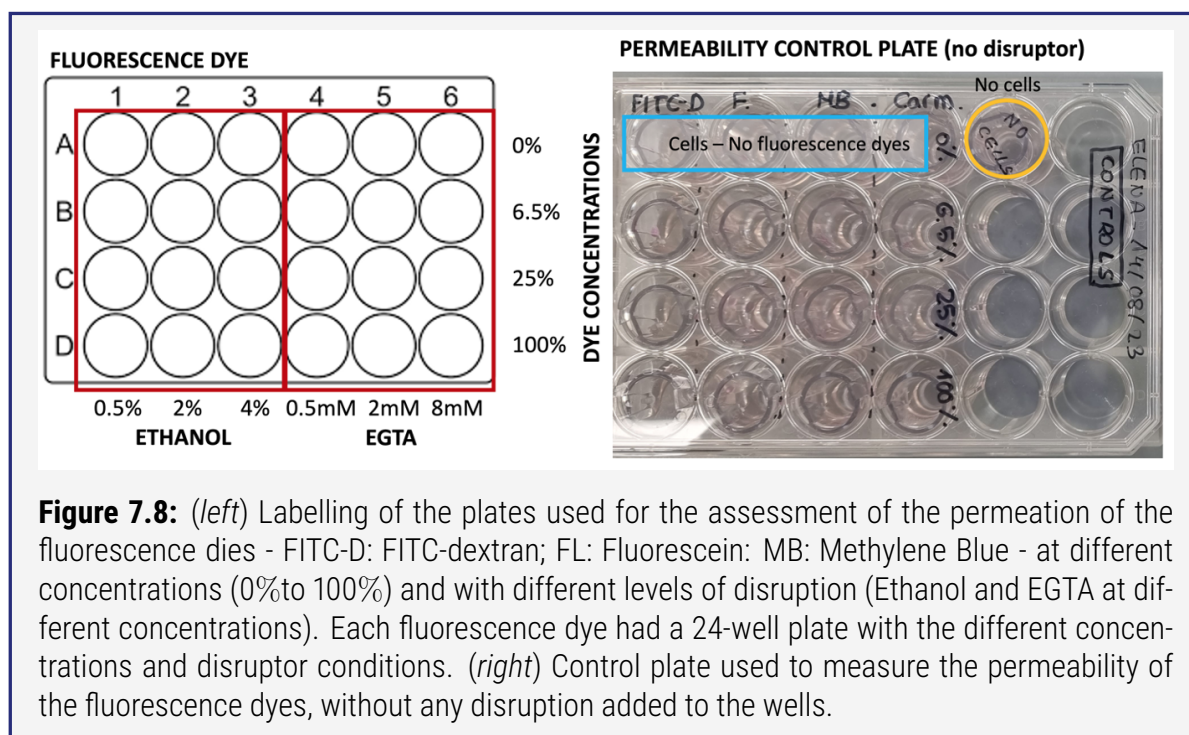


Figure 7.8: (left) Labelling of the plates used for the assessment of the permeation of the fluorescence dyes - FITC-D: FITC-dextran; FL: Fluorescein; MB: Methylene Blue - at different concentrations (0% to 100%) and with different levels of disruption (Ethanol and EGTA at different concentrations). Each fluorescence dye had a 24-well plate with the different concentrations and disruptor conditions. (right) Control plate used to measure the permeability of the fluorescence dyes, without any disruption added to the wells.

7.2.5 Protocols

Protocol 1: Thawing

1. Remove vial from the liquid nitrogen.
2. Loosen the cap of the vial slightly to release the pressure inside.
3. Transfer the vial into a 37°C water bath.
Keep the lid of the vial above the surface of the water to reduce the chances of contamination.
4. When cells are almost thawed (i.e., small remains of ice within the vial are visible), move the vial to a sterile station, such as a tissue culture hood.
5. Wipe the outside of the vial with a 70% ethanol solution and then, remove the lid.
6. Carefully, remove the cell suspension using a pipette and transfer the remaining content to a centrifuge tube and add 10ml of medium.

7. Spin the cells at ≈ 300 ppm for 5 minutes. Gently aspirate most of the medium, taking care not to aspirate the pellet of cells.
8. Add 10 ml of fresh medium and resuspend the cells to dissociate the pellet into individual cells.
9. Place them into a T25 flask and examine the suspension using a microscope.
10. Place the flask in a 5% CO_2 incubator.

Protocol 2: Passaging

1. Inspect the flask with MDCK cells, previously plated. Confluence of 80% (Fig. 7.3B) indicates cells are ready to be split again.
2. Aspirate the growth medium from the stock cell culture flask used for passaging.
Cells are attached to the base of the flask.
3. Add 5-10ml of PBS. Gently rotate the flask to wash the cells. Remove the PBS.
4. Add 3-5ml of 0.25% of Trypsin-EDTA to the flask to detach the cells from the flask. Tilt the flask to make sure cell surface is evenly coated. Return the flask to a 37°C incubator with 5% CO_2 .
5. After 5-8 minutes, take the flask from the incubator and shake it gently to detach the cells. Observe under the microscope.
If cells are properly detached, they will appear round and in suspension.
6. Rinse the flask with 5ml of growth medium to neutralize the trypsinization. Aspirate the suspension and pipet cells 3-5 times up and down to dissociate the suspension into individual cells. *This will yield $\approx 2 \times 10^6$ cells. However, number of cells should not be used as a routine guide, as morphology is a much better indicator of growth state.*
7. Use a 10ml serological pipette to collect the solution containing the cells and add them into a falcon tube.
8. Centrifuge the cells (≈ 1000 rpm for 5 minutes) and discard the supernatant.
9. Add 5ml of growth medium to the cells.

Follow steps 10 – 12 for seeding cells into flasks or steps 13 – 14 for seeding into transwell plates.

10. To seed them into a new flask, we use the following equation:

$$\text{Vol2} = \text{Vol1} \times \text{Split Ratio} \times \text{Volume ratio}$$

Based on the Split Ratio (1:10) and the type of flask (T25 or T75), we will take:

- $0.5\text{ml} = 5\text{ml} \times 0.1 \times 1$ (for a T25 flask and 1:10 split ratio).
- $1.5\text{ml} = 5\text{ml} \times 0.1 \times 3$ (for a T75 flask and 1:10 split ratio).

11. Add the calculated volumes (Vol_2) to the flasks and add 4.5 ml and/or 8.5 ml of growth medium for the T25 flask and T75 flask, respectively.
12. Label the flask, with the cell line (MDCK), split ratio (1:10) and number of passage and, place the flask/s in a 5% CO_2 incubator.

To maintain the functionality and quality of the cells, keep the number of passages <30 .

13. To seed the cells into the transwell, add $200\ \mu\text{l}$ of the solution containing cells and growth medium on the apical compartment. Then, add 1ml of growth medium (no cells) in the basolateral compartment.
14. Repeat previous steps as many times as transwell plates are needed. Include at least one transwell without cells to use as a blank. *For the blank transwell (no cells), add $200\ \mu\text{l}$ of growth medium (no cells) in the apical compartment and 1 ml on the basolateral compartment. Label it as blank/control.*

Protocol 3: TEER Measurements

1. Attach the electrodes to the EVOM3 and introduce them into a falcon tube with growth medium. Make sure the electrodes are covered with the solution. Select the resistance mode with units in ohms and 24 wells as the plate size.

This step ensures the EVOM3 is calibrated and ready for the measurements.

2. After 30 minutes, remove the transwell plate from the incubator and place it on a laminar flow hood.
3. Remove the lid and using sterile forceps (previously wiped with ethanol), rotate the transwells to keep them all in the same position.

This will speed the TEER measurements process.

4. Place the electrode into the first (control, no cells) transwell, with the short section of the electrode is in the apical (top) compartment and the long side is inserted into the basolateral chamber using the gaps between the insert and the well.

This measure establishes the baseline of the measurements ($TEER_{blank}$).

5. Maintain the position for a couple of seconds the let the EVOM3 to stabilise the reading. Then, save the value.
6. Extract the electrode carefully and wash it on the growth medium used before for the calibra-

tion.

This is done to prevent cross contamination between transwells, as some of them have different conditions in the apical chamber, such as fluorescence dye or disruptor concentrations.

7. Continue measuring the rest of the transwells.

If the transwell contains cells, make sure the electrode in the apical chamber is not in contact with the membrane, but ensure that it is sufficiently covered with the solution.

8. Once all the wells are measured, make sure to save the readings. Cover the plate with the lid, return it to the incubator and remove the next plate if needed.
9. After all the measurements are done for the day, wash the electrodes of the EVOM3 with 70% ethanol and water, alternating. Repeat this three times. Then shake the electrode on top of a absorbent tissue to dry it.
10. TEER values are calculated using the following equation:

$$TEER_{membrane} = TEER_{measured} - TEER_{blank}$$

TEER <0 represents damage in the cell monolayer barrier.

Protocol 4: Fluorescence contrast agents - maximum concentrations

For FITC-dextran: concentration of 100 µg/ml

1. Measure 5x100 µg (0.500 mg) of FITC-dextran from Sigma Aldrich - (CAS-number: 60842-46-8).
2. Add 5 ml of growth medium to the solution.
3. Spin the solution to ensure homogeneity.
4. Protect solution from light.

For fluorescein: concentration of 0.835 µg/ml

1. Measure 5x0.835 µg (4.175 µg) of fluorescein from Sigma Aldrich - (CAS-number: 518-47-8).
2. Add 5 ml of growth medium to the solution.
3. Spin the solution to ensure homogeneity.
4. Protect solution from light.

For Methylene blue: concentration of 1.461 µg/ml

1. Measure 5x1.461 µg (7.305 µg) of Methylene blue from Sigma Aldrich - (CAS-number: 122965-43-9).
2. Add 5 ml of growth medium to the solution.

3. Spin the solution to ensure homogeneity.
4. Protect solution from light.

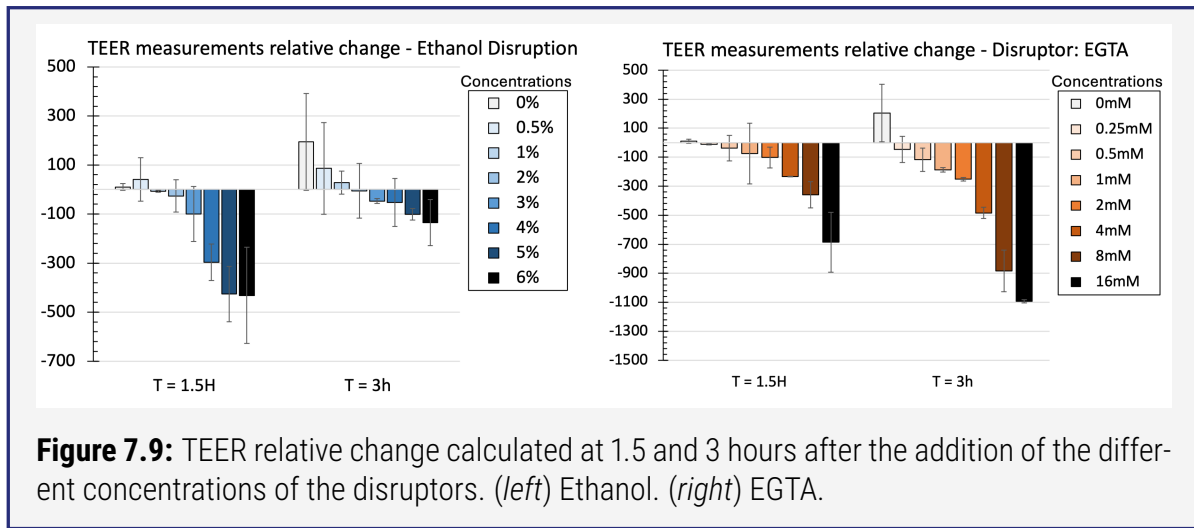
7.3 Results

7.3.1 Disruptor optimisation

As explained in Section 7.2.4.2, we first investigated the use of Ethanol and EGTA as epithelial barrier disruptors using different concentrations, shown in Table 7.2. After applying the disruptors into independent MDCK monolayer transwells, TEER measurements were collected at times 0 hours, 1.5 hours and 3 hours (see Fig. 7.9). The data has been extracted using the following equation:

$$TEER_{\Delta T} = TEER_{T'} - TEER_{T0h} \quad (7.8)$$

, where $T' = 1.5$ or 3 hours.



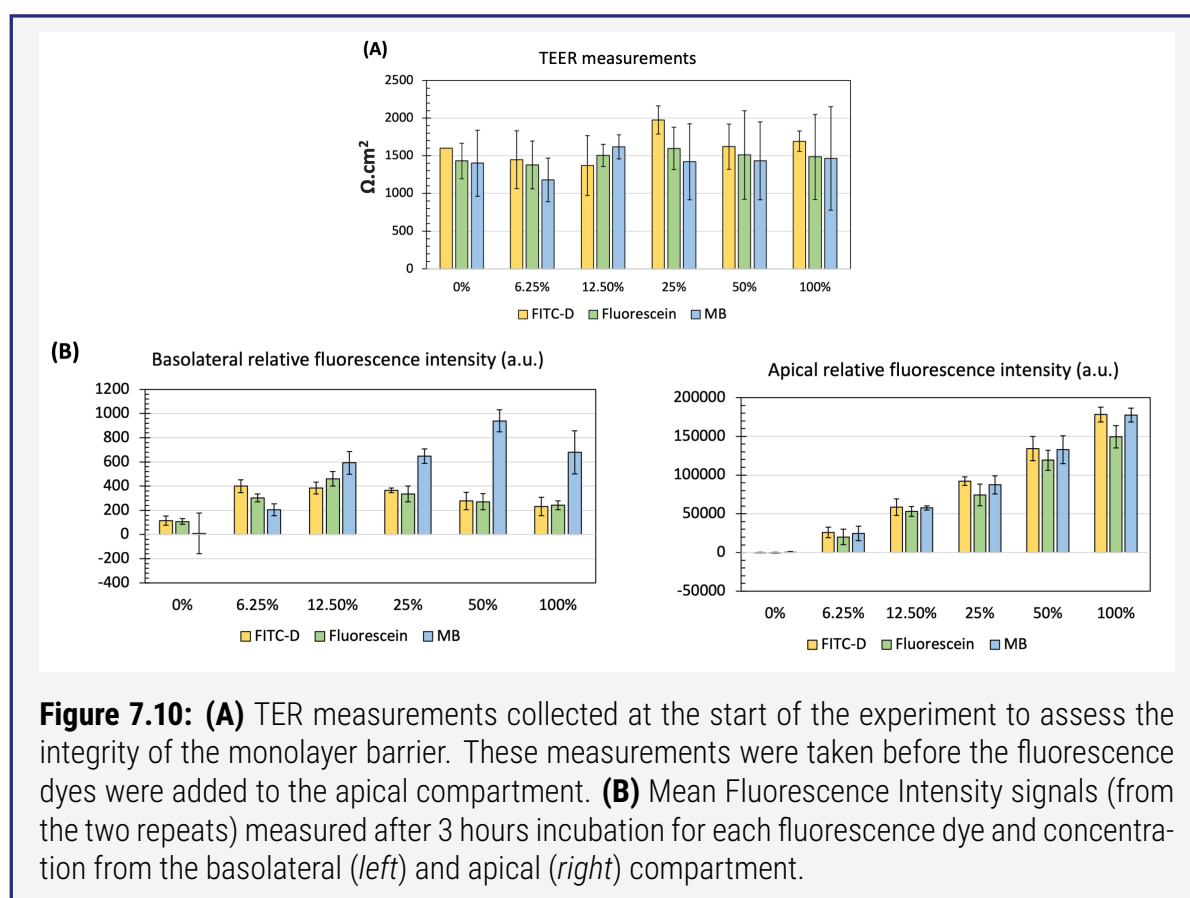
Based on the equation, a negative change in the TEER value indicates lower barrier resistance, which is indicative of epithelial barrier damage. As we can see in Fig. 7.9, control conditions, where disruptors were not applied (concentration of 0% and 0 mM for Ethanol and EGTA, respectively), show positive values. On the contrary, as expected, higher concentrations of Ethanol and EGTA produce larger changes in TEER values, with highest changes observed with EGTA 8 mM. However, even though both disruptors show similar effects on the epithelial barrier, it is worth noting that the impact of ethanol (after 3 hours) is not as pronounced as that of EGTA. This could be attributed to the evaporation of Ethanol, due to the small volumes used, causing less barrier damage over time (i.e., $TEER_{t=3h} > TEER_{t=1.5h}$). Overall, both disruptors showed an impact in the permeability of the barrier, which is primarily attributed to a disruption in the integrity of the tight junctions. Bootz-Maoz et al., for example, showed that EGTA selectively damages the tight junction's integrity, maintaining the viability and functionality of the barrier [274]. In the case of Ethanol, Ma et al. also showed that concentrations of ethanol below 10% damage the tight junctions of the epithelial cells, without causing any significant cytotoxicity [269]. Therefore, given the observations presented in

Fig. 7.9, concentrations of 0.5%, 2% and 5%, for Ethanol, and 0.5 mM, 2 mM and 8 mM, for EGTA, were used for the experiments in Section 7.3.4 to induce barrier damage at different stages: light, medium and severe.

7.3.2 Dye optimisation

In addition to investigating the effect of the disruptors, we also analysed the permeation of the dyes at different concentrations. By doing so, we could analyse the permeability of the different dye concentrations in normal conditions. The results will help to established what concentrations are going to be used in the permeability assays in Section 7.3.4.

As explained previously, MDCK cells were seeded in a 24-well plate and different concentrations of the fluorescence dyes – Fluorescein, FITC-dextran and Methylene Blue – were added to the apical compartment. The concentrations were prepared as explained in Section 7.2.4.3, labelled as 0%, 6.25%, 12.5%, 25%, 50% and 100%. Fig. 7.10 shows the TEER measurements, as well as the fluorescence intensity measured at the basolateral and apical compartments for each dye. TEER values measured at the start of the experiments showed values above 1000Ω , which indicate the formation of monolayers with strong tight junctions [285].



The values reported in Fig. 7.11 shows the different fluorescence intensities collected - after 3 hours of incubation - from each compartment (apical and basolateral) and fluorophore concentration. As expected, the more concentrated solutions provided higher fluorescence intensities. This can be observed in the values from the apical compartment in Fig. 7.11B (right). Given that no disruptor was added to the medium, it is expected that the strong tight junctions measured at $T = 0$ hours (Fig. 7.11A) are also present at the time the fluorescence measurements were made - after 3 hours incubation. This will explain the limited permeation of the fluorophores observed in Fig. 7.11B and Table 7.4. The fluorescence intensities observed in the apical compartment correlated with $\approx 90\%$ of fluorescence in the stock solutions for each concentration. In the basolateral side, due to the strong tight junctions, the permeation of the dyes was reduced significantly (p values < 0.01), with only around 1% of the stock solutions permeating through the membrane. This small permeation to the basolateral chamber was attributed to the natural translocation of the dyes through the paracellular pathway.

Table 7.4: Assessment of the permeation of the fluorescence dyes under normal (no disruption) conditions. Table shows the percentage of fluorescence intensity, relative to the stock solutions $[F_0]$, for the basolateral and apical compartment, for each fluorescence dye and concentration. Values were measured after 3 hours incubation. FITC-D - FITC-dextran; MB - Methylene Blue.

Panel A: Percentage of Basolateral fluorescence intensity relative to the fluorescence signal of $[F_0]$

Fluorophores concentration	FITC-D	Fluorescein	MB
6.25 %	1.422	1.399	0.801
12.5 %	0.636	0.831	0.978
25 %	0.388	0.441	0.714
50 %	0.205	0.225	0.684
100 %	0.129	0.160	0.376

Panel B: Percentage of Apical fluorescence intensity relative to the fluorescence signal of $[F_0]$

Fluorophores concentration	FITC-D	Fluorescein	MB
6.25 %	92.888	93.005	95.996
12.5 %	96.818	95.843	95.110
25 %	98.060	97.794	96.430
50 %	98.977	98.877	96.581
100 %	99.353	99.198	98.120

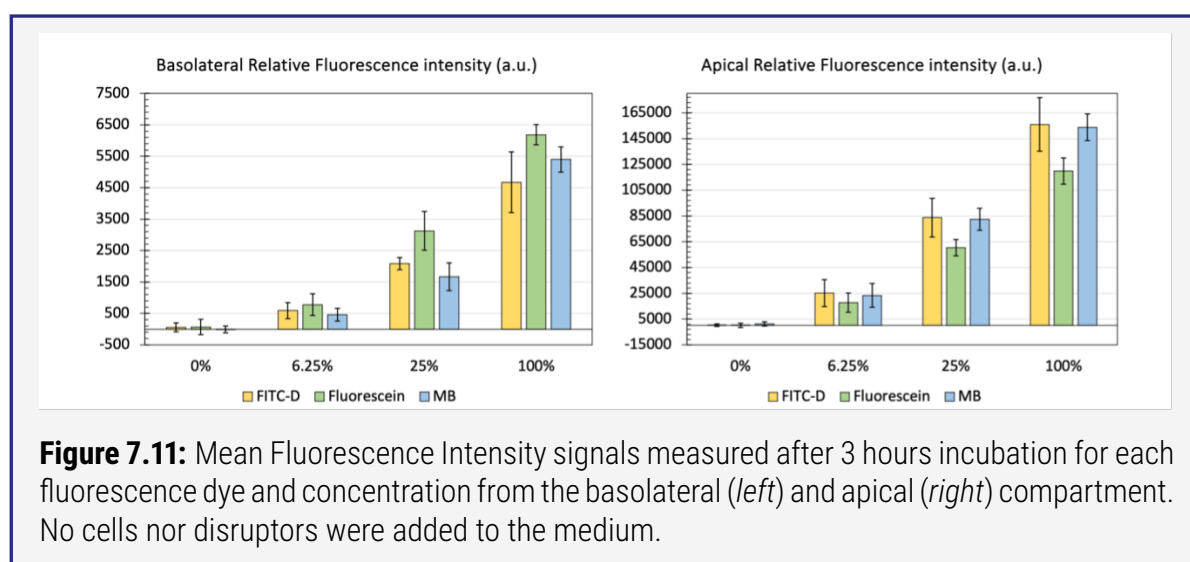
Interestingly for the Methylene Blue, at the basolateral compartment in Fig. 7.11B, an increase in the fluorescence signal is observed for the higher concentrations (i.e., 50% and 100%). However, this increase proved to be no significant (p value > 0.05) when calculating the fluorescence intensities relative to the stock concentrations (Table 7.4). Fluorescence values observed in Fig. 7.11B at concentrations 0% are produced by the autofluorescence of the growth medium from the api-

cal compartment that permeated into the basolateral compartment, increasing the fluorescence signal. However, this effect was considered when calculating the relative concentrations (Table 7.4). No saturation nor self-quenching effect was observed when measuring the fluorescence intensities from the apical compartment, even for the highest concentrations used.

After analysing these results, concentrations of 6.25%, 25% and 100% are expected to provide significant discrimination between the conditions. These concentrations are then used in Section 7.3.4 for the assessment of the permeability of the dyes under different conditions.

7.3.3 Transwell insert - control permeability

As explained previously, we hypothesised that the permeation of the fluorescence dyes is mainly produced through the paracellular pathway, mainly driven by the integrity of the tight junction of the epithelial barrier. In addition, we also expected that the permeation of the dyes is highly influenced by their molecular weight and, therefore, we also considered it important to analyse the potential impact of the insert membrane, with a pore size $0.4\ \mu\text{m}$, on the passive permeation of the fluorescence dyes. To do so, we added the fluorescence solutions into the apical compartment of the transwells inserts, this time without cells, and fluorescence intensity measurements were calculated after 3 hours from both compartments (apical and basolateral). The concentrations for the fluorescence dyes – 0%, 6.25%, 25% and 100% - were extracted from the previous experiment, in Section 7.3.2. TEER measurements were not collected in this case, as no cells were seeded in any of the plates. Fig. 7.11 shows the mean fluorescence intensity values measured at the basolateral and apical compartments for each dye.



As anticipated, higher concentrations result in increased fluorescence intensity in both compartments. Table 7.5 illustrates a significant difference between the control wells (with 0% dye concentration) and the other wells, indicating a permeation of the dyes through the membrane. In a dual compartment model, the permeation of the dye is expected to follow a diffusion process from the

chamber with higher concentration (apical) to the one with lower concentration (basolateral). This diffusion will continue until an equilibrium is reached, leading to similar substrate concentration (i.e., similar fluorescence intensity in this case) in both compartments. We expect that, in a model with no cells, the permeation of the molecules from the apical to the basolateral compartment occurs within a small time window (shorter than in the presence of a cell monolayer). Therefore, in this case, equilibrium is expected to be reached before or around 3 hours incubation. However, a significant difference is observed between the fluorescence intensity signals of the apical and basolateral chambers (p value < 0.05 , Table 7.5B). This observation suggests an influence of the filter membranes on the permeation of the dyes.

Table 7.5: Statistical analysis between the apical and basolateral compartments, as well as between the control wells (0% concentration of dye) and the rest of the concentrations. P -values from the paired Student t-test are shown. FITC-D - FITC-dextran; MB - Methylene Blue.

Panel A: P values from the comparison between the control wells (0% of dye) and concentrations

	Basolateral compartment			Apical compartment		
Concentration	FITC-D	Fluorescein	MB	FITC-D	Fluorescein	MB
6.25 %	0.1619	0.0179	0.1876	0.0158	0.0043	0.0929
25 %	0.0610	0.0247	0.0049	0.0316	0.0044	0.0219
100 %	0.0274	0.0144	0.0421	0.0368	0.0084	0.0041

Panel B: P values from the comparison between the apical and basolateral compartments

Concentration	FITC-D	Fluorescein	MB
6.25 %	0.0114	0.0063	0.0789
25 %	0.0338	0.0063	0.0191
100 %	0.0371	0.0094	0.0055

This hypothesis is further supported by the percentage of relative fluorescence intensity shown in Table 7.6. It reveals that less than 4% of the fluorescence dyes, initially added to the apical compartment, were detected in the basolateral side. This observation suggests the significant influence of the pore size of the membrane ($0.4 \mu\text{m}$) on the permeation of the dyes. This effect is particularly important for larger fluorescence dyes, such as FITC-dextran, with a molecular weight higher than 4k Da. In addition, it is worth mentioning that the charge of the fluorescence dyes can potentially impact their permeation. For example, positively charged dyes, such as fluorescein or FITC-dextran, tend to interact more readily with negatively charged cell membranes (such as the ones formed by MDCK cells [286, 287]), potentially facilitating their uptake. Methylene blue, on the contrary, is a negatively charged dye that may experience repulsion, resulting in reduced permeation. However, the interaction between the charge properties of the dye molecules and the MDCK cell membrane is complex, and further work is needed to fully understand these interactions.

Table 7.6: Assessment of the permeation of the fluorescence dyes after 3 hours (no cells)

Table shows the percentage of fluorescence intensity, relative to the stock solutions $[F_0]$, for the basolateral and apical compartments, for each fluorescence dye and concentration. FITC-D - FITC-dextran; MB - Methylene Blue.

Panel A: Percentage of Basolateral fluorescence intensity relative to the fluorescence signal from $[F_0]$

Concentration	FITC-D	Fluorescein	MB
6.25 %	2.093	3.599	1.806
25 %	2.213	4.114	1.838
100 %	2.606	4.103	2.987

Panel B: Percentage of Apical fluorescence intensity relative to the fluorescence signal from $[F_0]$

Concentration	FITC-D	Fluorescein	MB
6.25 %	89.535	82.003	90.972
25 %	88.935	79.428	90.812
100 %	86.968	79.486	85.063

In the results presented here, no cells were seeded in the transwell inserts, and only the impact of the membranes is analysed. We speculate that PET (polyethylene terephthalate) membranes do not have inherent charge properties that directly influence the permeation of the dyes. Instead, we assumed that PET membranes primary permeability effect is mechanical, based on their pore size and physical characteristics [288]. This is because we presume that the charge generated by the cell membranes has a significant higher impact in the permeation of molecules. We assumed that, when no cell are involved, a time window of 3 hours is enough to reach the equilibrium between the compartments. Therefore, the results presented in this section might suggest that the use of filters with higher pore size ($0.8\ \mu\text{m} - 3\ \mu\text{m}$) could potentially alleviate these permeability constraints. Nevertheless, due to the limitations in the consumables available for the experiments, we were compelled to proceed with the current membrane filters (PET with $0.4\ \mu\text{m}$ pores size). To facilitate the comparison of the permeation of the different dyes in these later experiments, relative concentrations are calculated based on the fluorescence signals observed in this section (Fig. 7.11), as they represent the maximum achievable permeation of the dyes.

7.3.4 Permeability assay

The previous sections have primarily examined the permeation of the different fluorescence contrast agents across different concentrations, alongside assessing the effect of Ethanol and EGTA on the integrity of cell monolayer models. In this final section we investigate the permeation of different fluorescence dyes – Fluorescein, FITC-dextran and Methylene blue – across 2D MDCK

monolayers with multiple permeability conditions induced by varying concentrations of the disruptors – Ethanol and EGTA. The experiments conducted here include the fluorescence concentrations at 6.25%, 25% and 100%, alongside Ethanol and EGTA disruptors at concentrations of 0.5%, 2% and 5%, and 0.5 mM, 2 mM and 8 mM, respectively. MDCK cells were seeded into 24-transwell plates, and the different fluorescence and disruptor concentrations were added to the apical compartments following the label depicted in Fig. 7.8. TEER measurements were collected at time 0 hours and 3 hours to analyse the effect of the disruptors. TEER relative changes were calculated using the following equation:

$$\Delta TEER = TEER_{relative} = TEER_{T=3h} - TEER_{T=0h} \quad (7.9)$$

Figures 7.12, 7.13 and 7.14 show the fluorescence intensity values and relative TEER ($\Delta TEER$) values for the fluorescence dyes: fluorescein, FITC-dextran and Methylene Blue, respectively. Across all conditions, the largest magnitudes of TEER changes ($\Delta TEER$) are observed at concentrations of 5% Ethanol and 8 mM EGTA, which are the highest concentrations used. Interestingly, Ethanol disruption exhibits a less pronounced and more unstable effect, resulting in minimal $\Delta TEER$ or occasionally positive $\Delta TEER$ values at 0.5% and 2% concentrations. This variability is primarily attributed to the Ethanol evaporation during the incubation period of 3 hours. Conversely, EGTA demonstrates a more substantial and consistent disruption effect across all cases, with the largest $\Delta TEER$ magnitudes recorded at -2809, -2036 and -3339 (using EGTA = 8 mM) for fluorescein, FITC-dextran and Methylene Blue, respectively.

These results highlight the efficacy of EGTA in compromising the integrity of the cell monolayers, providing different levels of disruption: mild (0.5 mM), moderate (2 mM) and severe (8 mM). Despite the potential limitation of the Ethanol evaporation, the concentrations used also provided distinct levels of disruption, with the most significant difference observed at 5% concentration. As expected from the TEER results, higher fluorescence intensities are observed on the basolateral compartment when cell monolayers were incubated with EGTA. This is associated with a higher permeation of the dyes from the apical to the basolateral compartment, as illustrated by the mean percentages of fluorescence signal, relative to the stock solutions, shown in Table 7.7, 7.8 and 7.9. It is important to mention that values presented in these tables also take into account the reference values (i.e., maximum permeation) from Section 7.3.3, where permeation of the dyes was measured without the presence of cells - and so variability in the percentage of maximum permeation is expected.

Fluorescein exhibited the highest mean percentage of permeation ($\approx 100\%$) when incubated with 8 mM EGTA. FITC-dextran and Methylene blue, under the same condition, showed mean percentage of permeation of 70.386% and 24.298%, respectively. In the case of cell incubated with 5% Ethanol, similar permeation of fluorescein and FITC-dextran are observed, with maximums no higher than 33.905% and 24.298%, respectively due to potentially high fluorescence Methylene Blue, taken up by living cells, might be undetected in either of the compartments. In

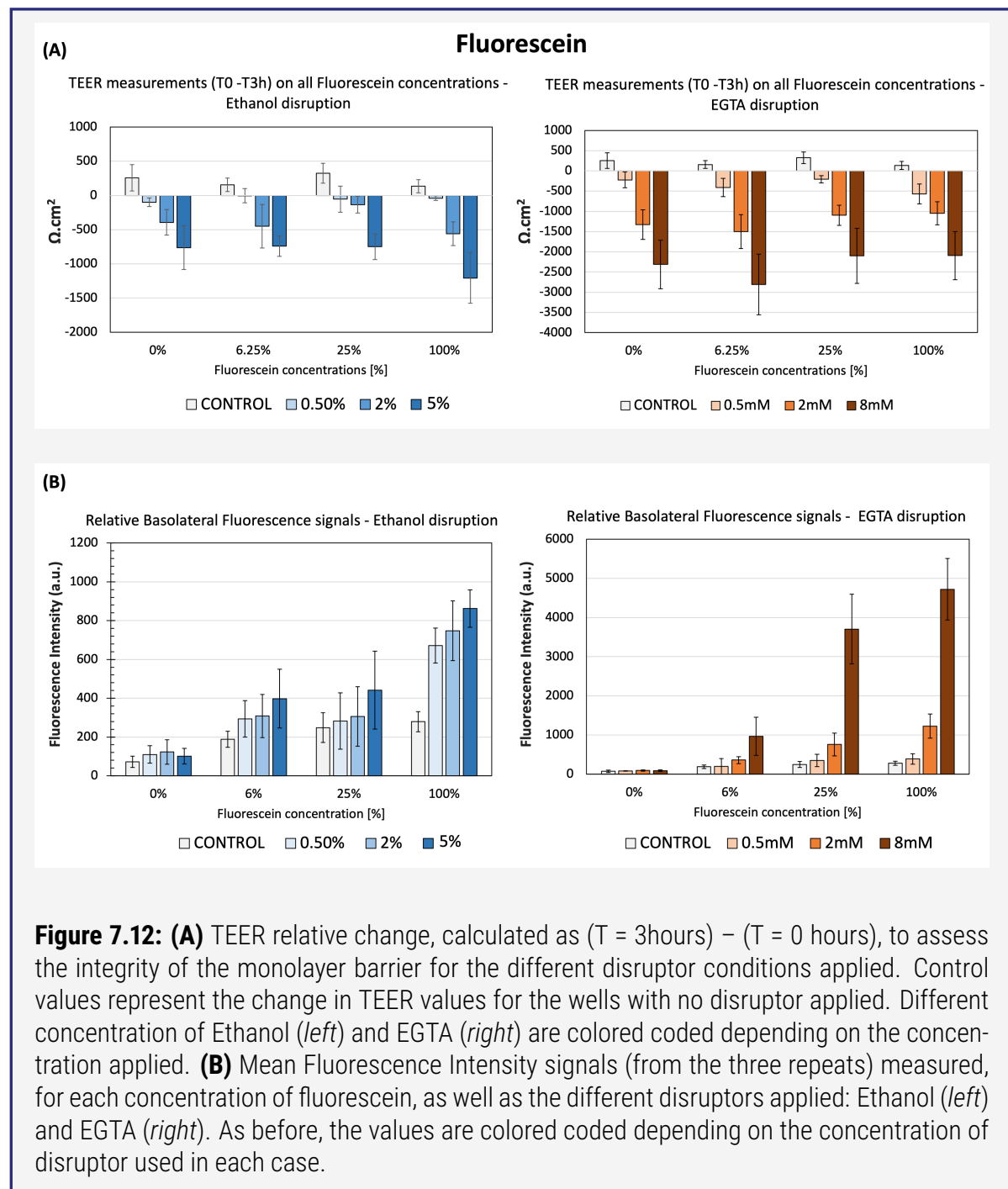


Table 7.7: Percentage of *FLUORESC EIN* fluorescence intensity, relative to the stock solution [F_0], for the basolateral compartment for the different concentrations of fluorescein and disruptor.

Panel A: Ethanol

Concentration	0.5 % Ethanol	2 % Ethanol	5 % Ethanol
Fluorescein 6.25 %	35.927	48.194	67.516
Fluorescein 25 %	8.910	12.616	18.149
Fluorescein 100 %	10.702	14.203	16.050
Mean	18.513	25.004	33.905

Panel B: EGTA

Concentration	0.5 mM EGTA	4 mM EGTA	8 mM EGTA
Fluorescein 6.25 %	27.163	50.179	119.439
Fluorescein 25 %	10.819	28.647	106.837
Fluorescein 100 %	6.923	29.695	98.555
Mean	14.968	36.174	108.277

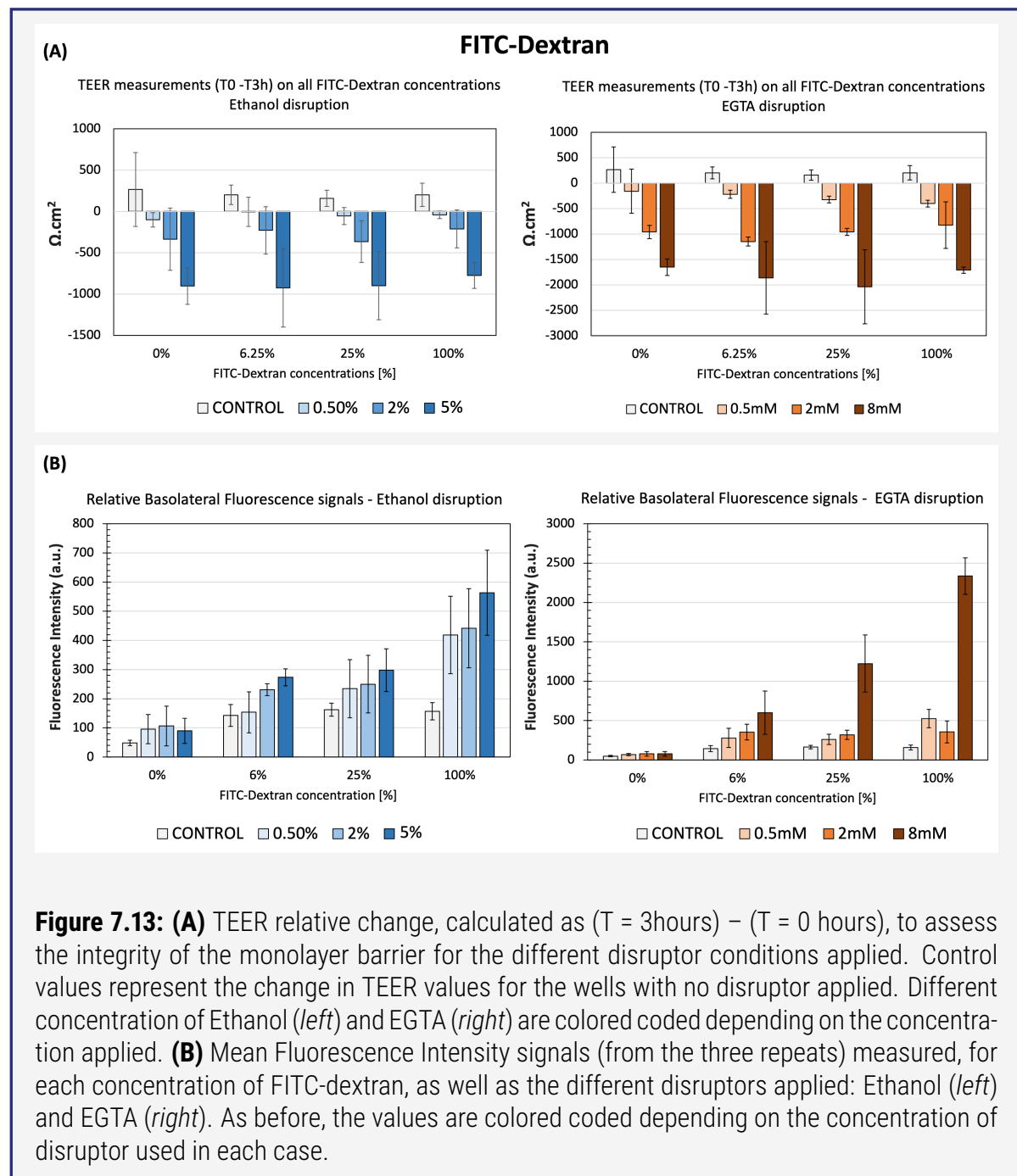


Table 7.8: Percentage of FITC-Dextran fluorescence intensity, relative to the stock solution, for the basolateral compartment for the different concentrations of FITC-Dextran and disruptor.

Panel A: Ethanol

Concentration	0.5 % Ethanol	2 % Ethanol	5 % Ethanol
FITC-D 6.25 %	26.163	39.390	46.531
FITC-D 25 %	11.269	12.005	14.312
FITC-D 100 %	8.957	9.456	12.053
Mean	15.463	20.283	24.298

Panel B: EGTA

Concentration	0.5 mM EGTA	4 mM EGTA	8 mM EGTA
FITC-D 6.25 %	47.431	60.231	102.427
FITC-D 25 %	12.531	15.311	58.774
FITC-D 100 %	11.254	7.607	49.959
Mean	23.738	27.716	70.386

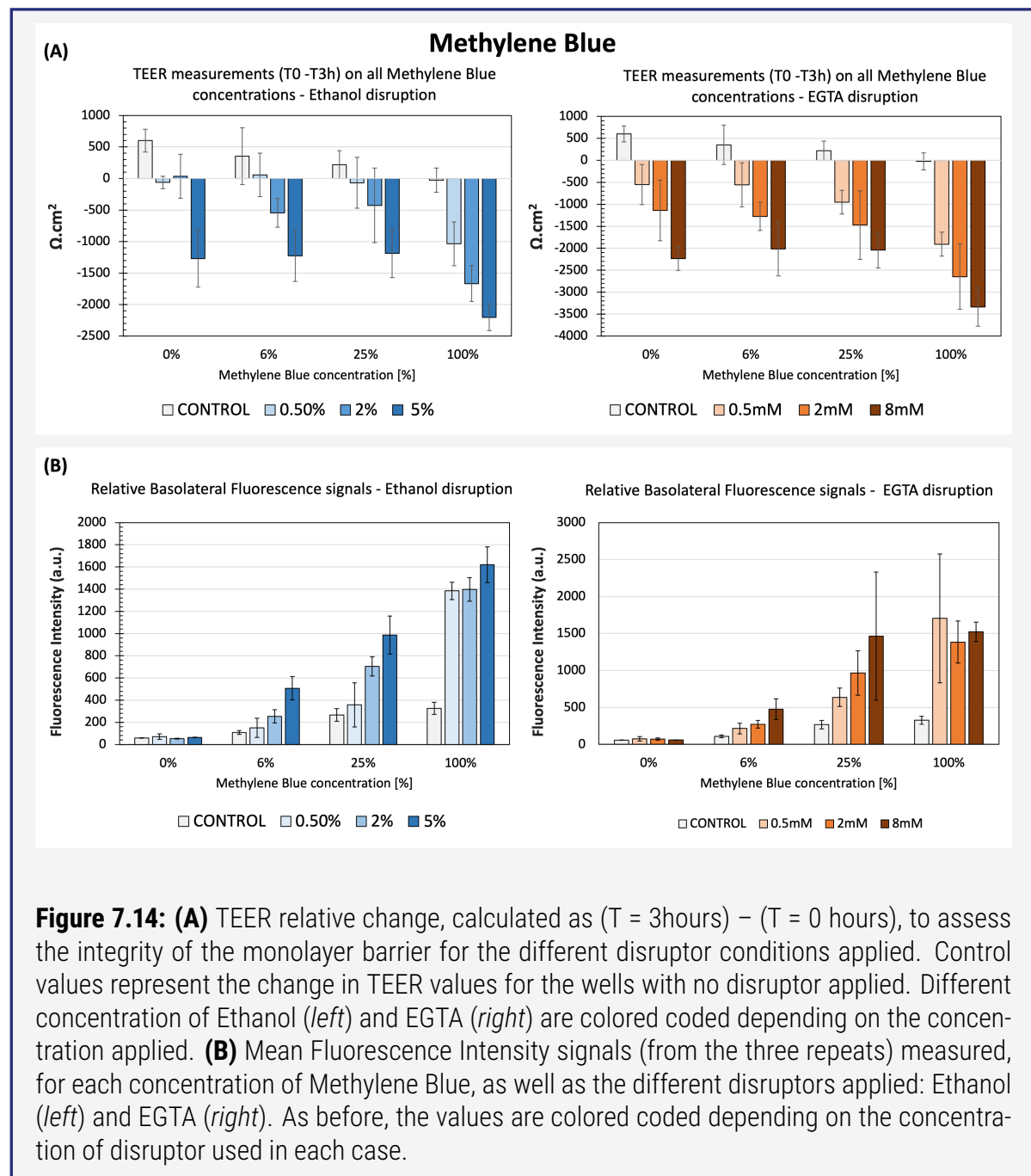
Table 7.9: Percentage of Methylene Blue fluorescence intensity, relative to the stock solution, for the basolateral compartment for the different concentrations of Methylene Blue and disruptor.

Panel A: Ethanol

Concentration	0.5 % Ethanol	2 % Ethanol	5 % Ethanol
MB 6.25 %	32.635	54.875	109.793
MB 25 %	21.402	42.216	59.237
MB 100 %	25.626	25.871	30.003
Mean	26.554	40.987	66.343

Panel B: EGTA

Concentration	0.5 mM EGTA	4 mM EGTA	8 mM EGTA
MB 6.25 %	46.592	58.723	103.448
MB 25 %	38.219	58.073	87.793
MB 100 %	31.573	25.622	28.187
Mean	38.794	47.472	73.142



7.4 Conclusion

Fluorescein is a fluorescence contrast agent widely used in the clinic, as well as in research, demonstrating its effective permeation through membranes [146, 186, 206, 222]. Previous chapters have demonstrated the use of oral administered fluorescein as a relevant fluorophore for the transcutaneous assessment of changes in gut permeability. The non-invasive detection of fluorescein through the skin, across a wide range of skin tones, offers significant advantages over other widely known fluorophores such as FITC-dextran [90]. This observation was further supported by the significant difference in fluorescein intensities observed between healthy and undernourished participants. However, fluorescein has also been shown to permeate easily even under permeability conditions self-categorised as “healthy”. This observation prompted further evaluation of fluorescein permeation across different gut permeability conditions. However, the complexity of the gut barrier presented challenges, as processes such as gastric emptying and fluorescence clearance occur alongside the permeation of fluorescein. Therefore, MDCK cell monolayer models were used to characterise fluorescein permeation under different barrier conditions in a more controlled environment. In addition, fluorescein permeation was also evaluated against FITC-dextran and Methylene Blue, widely used fluorescence dyes with different molecular sizes and fluorescence spectra.

For the disruption of the epithelial barrier, EGTA and Ethanol at different concentrations were used. Ethanol exhibited a less pronounced and unstable effect, attributed to its overtime evaporation during the 3 hours long experiment. EGTA, on the contrary, generated more consistent disruption of the cell monolayers. However, overall, both disruptors compromised the monolayer epithelial barrier by affecting the integrity of the tight junctions, leading to translocation of the fluorescence molecules from the apical to the basolateral compartment.

We also analysed the impact of the transwell inserts filters on the permeation of these dyes, prior to their evaluation, and found that the 0.4 μm pore size inserts were potentially limiting the permeation of the fluorescence dyes. This limitation was taken into account when the permeation of the different fluorescence dyes was analysed.

Fluorescein, FITC-dextran and Methylene Blue have different molecular sizes and therefore, permeability rates are expected to be different. In the case of Methylene Blue, a significant decrease in the TEER values, together with an increase in its relative permeation, indicated a potential toxicity effect from Methylene Blue at a concentration of 1.461 $\mu\text{g/ml}$. In addition, given the capability of Methylene Blue to permeate inside living and dead cells, interpretation of the results became more challenging. Therefore, for the purposes of this chapter, we focused on comparing the permeation of fluorescein and FITC dextran.

The results demonstrated that fluorescein, and also FITC-dextran, permeate through the paracellular pathway at different rates depending on the level of epithelial disruption, as expected from our

findings in previous chapters. More importantly, fluorescein permeation showed to be more distinct among the different stages of disruption, suggesting a greater sensitivity to different levels of epithelial damage, in comparison with FITC-dextran.

As explained previously, the investigation of the fluorescein permeation in cell monolayer models was partially driven by the high fluorescence intensities observed in specific participants in chapter 5 and 6. However, overall, the findings from this chapter support fluorescein as an effective marker of barrier integrity (at the levels of disruption tested here). In addition, the ability of fluorescein to be transcutaneously detected makes fluorescein a valuable indicator for non-invasively assessing gut barrier integrity. Finally, the results from this chapter also indicate that fluorescein may provide a low-cost alternative to FITC-dextran for future in vitro cell monolayer experiments (where typically FITC-dextran is used)

Chapter 8

Conclusion and future work

The primary focus of the thesis is to demonstrate the potential of a developed compact transcutaneous fluorescence sensor, combined with the use of fluorescein oral administration, to effectively assess changes in gut permeability non-invasively. The lack of standardisation between dual-sugar permeability tests (such as Lactulose:Mannitol or Lactulose:Rhamnose tests), their high cost, limited clinical translation as well as the invasiveness of procedures such as endoscopic biopsies, proved the need of more cost-effective, more robust and less invasive approaches to assess gut permeability. Therefore, the work presented in this thesis hypothesised that the developed compact fluorescence sensor can significantly improve the assessment of gut permeability and enable more widespread deployment of transcutaneous fluorescence spectroscopy as a tool for non-invasive assessment of gut function.

The following sections summarise each of the chapters presented in this thesis, excluding Chapters 2 and 3. The first section includes a concise overarching summary about the main findings of this thesis. Subsequent sections delve deeper into each chapter, with a focus on the potential future steps towards the improvement of the technology and the assessment of gut permeability.

8.1 Summary

Through comprehensive in vivo validation and subsequent data analysis, this thesis first demonstrated the potential of transcutaneous fluorescence spectroscopy, combined with the oral administration of fluorescein, to assess gut function. These findings supported the development of a compact and portable fluorescence sensor, capable of reliable and robust detection of changes in gut permeability, even with variations in fluorescence dose, optical conditions, and motion artifacts.

Results from these experiments showcased the cost-effectiveness, reliability and improved clinical adoption of the compact sensor compared to clinically validated spectroscopy systems, enabling further deployment in larger clinical studies. Despite the complexity of the study, the compact fluorescence sensor demonstrated significantly better discrimination between groups with different gut permeability conditions in comparison with current clinical approaches (i.e., Lactulose:Mannitol, Lactulose:Rhamnose, and endoscopic biopsies), reinforcing the potential of this approach. Finally, 2D cell monolayer models revalidated the use of fluorescein as a marker of gut permeability, demonstrating a higher dynamic range than FITC-dextran or Methylene Blue when analyzed under different levels of barrier dysfunction.

Overall, these findings validate the initial hypothesis that the use of transcutaneous fluorescence spectroscopy can significantly improve the monitoring and diagnosis of gut permeability disorders, offering improved cost-effective, robust, non-invasive, and real-time monitoring capabilities compared to current methodologies.

8.2 Computational analysis of fluorescence spectroscopy data

8.2.1 Algorithm development for automatic processing and analysis of fluorescence spectroscopy data – proof-of-concept

Chapter 4 has reported a series of analysis techniques for assessing changes in gut permeability. Transcutaneous fluorescence data from 11 healthy participants was recorded in two different scenarios. Firstly, transcutaneous fluorescence data was collected from the participants to measure their normal gut permeability by ingesting a dose of 500mg of fluorescein diluted in water. In the second experimental scenario, data was collected from the same participants after they were asked to drink a fluorescence solution (with the same fluorescein concentration as before) with an additional sugar content that induced an increased in gut permeability. As explained previously, Chapter 4 then focused on investigating different analysis approaches to characterise the fluorescence signals from each experimental condition.

The first analysis approach focused on extracting a series of parameters through different methods - from manual to more automatic - based on Peak Selection. Two additional parameters were also calculated - Peak AUC/Slope and Peak AUC * Peak Time - as a simplified approach to correct for any variability in the gastric emptying rates between participants. Comparison of all the extracted parameters - Peak Time, Peak Intensity, Peak AUC, Peak AUC/Slope and Peak AUC * Peak Time - provided significant difference (p value < 0.05) between the datasets, allowing for an effective, simple and quick strategy that can be implemented within the technology to differentiate between the two stages of gut permeability.

Given the potential impact of the gastric emptying rate on the dynamics of the signal, the following work then focused on further improving the correction of the fluorescence signals, this time, for the elimination of the fluorescein from the body. To do so, two exponential decay fitting approaches were implemented to extract the decay rates from each signal. However, results from the decay fitting models showed high dependence on the selected starting points, which, together with the absence of clear exponential decay regions in some signals, suggested an inaccurate prediction of the elimination rate and thus unrealistic corrections. Future work regarding this topic is discussed later in this section and focuses on the prediction of the entire fluorescence signal using pre-defined equations, such as the method presented in Let et. al [145].

Phasor analysis was used to analyse the fluorescence signals in the frequency domain, in which data from all 11 participants was compressed into two frequency components: real and imaginary parts. These parameters were then integrated inside a support vector machine (SVM) algorithm to build a classification model that was tested using a leave-one-out methodology. The classifier provided an accuracy of 83.333%, which suggested that the extraction of phasor components followed by SVM has the potential to differentiate between stages of gut permeability in a fully automatic strategy.

Overall, results from this chapter demonstrated the capability of two different strategies - a simple and semi-automatic method through Peak Selection and a more complex and fully-automatic approach through Phasor Analysis - to differentiate between states of gut permeability. In addition, the significant difference found between the datasets when implementing these approaches reveals the potential of transcutaneous fluorescence spectroscopy for non-invasive assessment of intestinal permeability. This is of high importance for this thesis as the observations from this chapter suggested that transcutaneous fluorescence spectroscopy has significant advantages over existing permeability tests (e.g. L:M tests, Cr-EDTA assays, etc.) in terms of both invasiveness and time to report results, and the implementation of strategies such as the ones here presented implies the potential for rapid clinical translation.

Some of the limitations associated with the technology were addressed in the following chapters, including the development of a compact and portable system to further validate the technique in larger populations, with different skin types and fluorescein doses (Chapter 5), as well as in other

pathological conditions (Chapter 6). Immediate work is now focused on collecting more data to further validate the technology.

There are of course limitations associated with the analysis strategies presented in this chapter. For example, the use of simple, but rapid, analysis strategies, such as the manual Peak selection increases the probabilities of bias from the user that can lead to unreliable classifications and interpretation of the results. In the case of fully automatic approaches, such as the Phasor analysis, the classification is independent on the user input, but it might become less intuitive to interpret the results. A trade-off would be the use of automatic time domain approaches, similar to the automatic Peak Selection or deep learning algorithms such as long short-term memory (LSTM) network. In this case, improved strategies to reduce the impact of noise, such as fitting methods, and the collection of more data will be beneficial.

For the correction for the elimination of the dye from the body (i.e., decay rate), future immediate work should be directed toward refining the exponential decay fitting algorithms by incorporating additional features and constraints. Some of these strategies could involve the use of equations to describe the full curve, similar to the method described in Lett et. al [145], as well as the use of specific ranges for the expected decay rates. In addition, the selection of specific segments within the decay region of the signals will also help to focus the algorithm on regions with clear decay rates. Furthermore, exploring addition features, such as the uptake of the fluorescence intensity, could also improve the characterisation of the fluorescence curves and facilitate the discrimination between gut permeability states. Additionally, fluorescence signals with no clear decay region could be improved by recording data for prolonged periods (i.e., more than 3 hours). However, there is the risk of the technology becoming more time-consuming and uncomfortable for the participant. Finally, collection of blood samples and fluorescence data simultaneously could facilitate quantitative measurements of the concentration of translocated fluorescein and give information about the clearance rate of fluorescein, which could be used to correct the fluorescence signals. Considering this approach, correlation between gut permeability and fluorescence intensity signals in plasma could benefit the development of point-of-care technologies that could be use in larger populations for the rapid assessment of gut permeability.

Overall, the results presented in this chapter, along with any further data collected, will be used to guide the development of more advance processing and analysis tools (in terms of, for example, machine learning algorithms or fitting strategies) with the objective to improve the technology, allowing for effective and accurate assessment of changes in gut permeability.

8.3 Development and characterisation of a compact fluorescence sensor

Chapter 5 focused on the development of a compact transcutaneous fluorescence sensor that integrated the functionality of the system presented in chapter 4 (referred to as benchtop system) for the non-invasive assessment of gut permeability. The sensor provides measurements of fluorescence intensity of the absorption and elimination of fluorescein in the bloodstream after oral ingestion. In this case, the objective was to develop a more compact sensor that was suitable for deployment in wider clinical studies, including low-income settings. To this end the device is housed in a laptop-size structure for ease of transportation. In addition, it can be fully controlled automatically through an in-house written LabVIEW software. The device was designed and developed for ease of portability and low-cost and to make it safe, practical and comfortable for the user and the participant.

Having described the design and development of the sensor, chapter 5 also presented the results from the *in vitro* and *in vivo* validation of this system. The protocol focused on comparing the fluorescence intensity signals between the device and the control measurement (benchtop system) from, initially, fluorescence samples to later be deployed in clinical studies for further *in vivo* validation. To date, the sensor has been involved in 59 *in vivo* measurements of fluorescein intestinal permeation, in two different clinical studies (including chapters 5 and 6).

The preliminary *in vitro* validation measured the fluorescence signal from both the sensor and the benchtop system on different samples with fluorescein concentrations similar to those found in a realistic *in vivo* scenario. Results from these measurements showed good agreement between the sensor and the benchtop system, with a correlation coefficient of 0.983 (p value = 0.003). Further *in vivo* validation focused on measuring the fluorescence intensity signals this time from healthy participants after oral administration of 500 mg of fluorescein. Results from this *in vivo* validation exhibited a significant correlation (p value < 0.001) between the sensor and the control system, with a Pearson's correlation coefficient of 0.989. The following experiments focused on characterising the performance of the sensor under different conditions. These included variations in the contrast agent dose ingested by the participants as well as different skin tones. A potential linear relationship was found between the ingested dose (500 mg or 200 mg) and the observed fluorescence intensity after comparing the fluorescence intensity signals from both conditions. In addition, when characterising the sensor's response under different skin tone, results indicated minimal impact of the skin tone on the sensor's performance. Altogether, results demonstrated the potential of the developed compact sensor for assessing the permeation of fluorescein from the gut to the bloodstream transcutaneously. Overall, the sensor presented in this chapter showed promising performance for the assessment of changes in gut permeability, which prompted the deployment of it on a wider clinical study (discussed in Chapter 6). Considering this, further work

was focused on miniaturisation the sensor by designing a PCB electronic configuration that was later integrated in a smaller version of the sensor to facilitate deployability. This miniaturised version is now being deployed in different *in vivo* studies to measure gut permeability in conditions such as IBD or undernourished children [292].

Future work in this field is directed towards the improvement of some of the limitations found during the design, development and validation of the sensor presented in this chapter. Firstly, the design and development of the system was limited to the resources available. For example, the use of National Instruments (NI) DAQs allowed for a full control of the device via a customisable interface, ensuring accurate and precise data acquisition. However, this limited the miniaturisation and cost of the sensor. Considering this, microprocessors or microcontrollers such as Raspberry Pi or Arduino, with specific requirements, could be good alternatives to the use of NI DAQs.

When developing the sensor, the cost of the technology was reduced significantly, decreasing from $\pounds \approx 10\text{k}$ for the benchtop system to $\pounds \approx 2.2\text{k}$ for the sensor. As discussed, the use of a NI DAQ played a major role in the final cost of the developed sensor, along with the integration of a fibre probe. This probe allowed a maximum separation of $200\text{ }\mu\text{m}$ between the PDs and the LED. Without the optical fibre, the configuration of these optical sensors, when placed on a PCB, achieved only a minimum separation of 2.8 mm , which in previous experiments proved inadequate to detect fluorescence signals transcutaneously. Nonetheless, this limitation could potentially be addressed using customized PDs and LEDs that are in-house developed (or "bare die"). An alternative approach involves the use of light pipes to guide the light from the optical sensors to specific locations, allowing a higher separation between the PDs and the LED, while maintaining a light beam spacing of $200\text{ }\mu\text{m}$. Nevertheless, the effectiveness of the light transportation through these pipes needs further investigation.

In terms of the validation experiments, the performance of the sensor was characterised in two different fluorescein doses of 500 mg and 200 mg . However, validation under multiple doses, such as 25 , 50 and 100 mg will provide information regarding the limit of detection of the sensor and the sensitivity of the technology to lower doses, particularly important for children. In addition, because the sensor relies on the optical properties of the fluorescence light to assess the fluorescein permeation, a large number of participants with wider diverse skin tones and a robust categorisation different to Fitzpatrick scale, will further validate the potential of the system to deploy in large populations.

The last section of the chapter showed the steps taken towards further miniaturising the device. Ideally, the ultimate goal is to design a wearable device that participants could use comfortably without interfering significantly in their daily live and that could also be used in children. Considering this, other favourable experiments could include the investigation of the impact from natural movements of the participants, such as walking or executing daily tasks, on the fluorescence signals. This will provide information about how natural movements could affect the signals so

improvements can be made within the device. At the same time, the investigation of other parts of the body, instead of the fingertip, could also help to improve the comfortability of the sensor towards its use in the clinic, especially in children. As discussed, the next generation of the device is now being deployed in clinical studies in which some of these aspects will be investigated.

8.4 Clinical validation of a compact fluorescence sensor

8.4.1 Monitoring gut permeability in healthy and undernourished populations – a comparative study

Following from the work presented in Chapter 5, Chapter 6 focused on further investigating the fluorescence intensity profiles of undernourished adults in Zambia and to compare this data against healthy volunteers from the UK. To do so, a second sensor, a replica of the sensor presented in Chapter 5, was developed and later deployed in Zambia. The performance of the replica sensor was, initially, evaluated against the original sensor (referred to as Sensor 1) to establish the differences between the devices. This was carried out using fluorescence reflectance standards that allowed the calculation of a calibration ratio that was implemented on Sensor 2. The calibration of the systems allowed for a realistic comparison between the fluorescence datasets from both devices, particularly important for the clinical interpretation of the results from both studied groups.

In this case, the protocol used to collect the fluorescence signals in both groups was similar to that described in Chapter 5 and involved the oral ingestion of 200 mg of fluorescein in empty stomachs and the following collection of fluorescence signals using Sensor 1 in the UK and Sensor 2 in Zambia for at least 180 minutes. In addition, L:M and L:R samples were also collected from the healthy and undernourished groups, respectively. Despite the limited data available and the lack of standardisation of the dual sugar permeability tests, the initial comparison demonstrated significant correlation between the fluorescence intensity signals and the permeability tests for both groups. This was attributed to the similar molecular size between fluorescein and Lactulose and the permeability pathway followed by both molecules [125, 206]. As discussed in previous chapters, undernutrition is also characterised by an increased in gut permeability. Therefore, a difference between the healthy and undernourished groups was expected. When analysing the fluorescence intensity signals, a wide range of intensity variations was found in both groups which was considered normal among individuals and across populations. This variability resulted in a significant overlapping between the datasets with no significant differences found with respect to the time at which the fluorescence signals reached the maximum intensity (i.e., Peak Time). However, comparison of the peak intensities and Total AUC values from both groups exhibited significant differences (p values < 0.05), suggesting that undernourished participants had an increased permeation of fluorescein with respect to the healthy group. These results, therefore, showcased the potential of the

developed sensor for non-invasively assessing changes in gut permeability using transcutaneous fluorescence spectroscopy.

In addition, morphometry data was also collected from participants from Group 2 (Zambia) and later compared against the fluorescence signals and the *L:R* values. No significant correlation was found between the morphometry data and the fluorescence or dual sugar permeability data. Although limited data was available for the comparison, these results suggested that the morphometry data might only be sensitive to localised lesions within the location of the biopsies. Data from the fluorescence or sugar permeability tests, on the contrary, might represent better changes that occur across a larger surface in the small intestine. These observations, therefore, suggest that transcutaneous spectroscopy could be used as a complementary tool for assessment of changes in gut permeability and not a replacement of the biopsies.

Interestingly, further comparison between the percentage of Lactulose from both groups showed no significant difference, which was attributed primarily to the lack of standardisation between the protocols (i.e., different urine collection times, concentration of Lactulose and no conservative or stabiliser used in the samples). In addition, from a total of 80 participants from the undernourished group that were recruited, only *L:R* data from 42 was available due to human errors during the implementation of the protocol. All these observations highlight even further the need for more robust approaches to assess gut permeability.

To further demonstrate the potential of the fluorescence signals to discriminate between different gut permeability conditions, phasor analysis (presented in Chapter 4) was performed. The frequency components of the signals from both groups were then integrated into a *SVM* model that was able to classify the data with an accuracy of 80%.

In conclusions, in Chapter 6 we demonstrated the potential that transcutaneous fluorescence spectroscopy has over current clinical approaches to differentiate between different stages of gut permeability. In addition, the development of a second sensor allowed the implementation of the technology in a clinical study in two different countries at the same time, including a low-income setting. This chapter has demonstrated the advantage that the developed sensors has over the previous approach (i.e., benchtop system) in terms of friendliness, cost-efficiency and portability.

Future work is now focused on aspects of the protocol used during the clinical study as well as the further improvement of the device. As mentioned in the previous section, a miniaturised version of the devices presented in Chapter 5 is now going to be deployed in clinical studies for the assessment of changes in gut permeability in *IBD* patients and undernourished children. Therefore, the future work regarding the device is further explained in the previous section. In this section we focus on aspects that will help to improve future clinical studies centred in the assessment of changes in gut permeability using transcutaneous fluorescence spectroscopy. For example, a longitudinal study would provide information about changes over time in the fluorescence profile related to physiological factors, such as gastric emptying rate, diet or pathological conditions, in-

cluding the response to treatments. In addition, monitoring other factors, such as exercise, sleep or medication, along with the collection of fluorescence signals could provide information about confounding factors that could impact the gut permeability. Consistent with these ideas, during the analysis of the fluorescence signals, variations in the gastric emptying rates between participants were often suggested to impact the comparison between datasets. Correlation between oral administration of paracetamol and transcutaneously collection of fluorescein signals showed that fitting models can be used to measure the gastric emptying rates on healthy volunteers [145]. This suggest that a fully characterisation of the fluorescein profiles using advance signal processing approaches, as explained in 8.2.1, could be beneficial. More importantly, larger clinical studies with well-defined and more robust protocols will provide valuable information about gut permeability in different aspects of the pathogenesis, reducing the risk of associated errors or miss interpretations.

In conclusion, the results presented in this chapter demonstrated the potential of the technology to discriminate between different stages of gut permeability and will be used to guide further clinical studies, with a particularly focus in undernourished children. The aim is to investigate the full potential of this technology, while improving the clinical assessment of gut permeability.

8.5 Alternative fluorophores for gut permeability assessment

8.5.1 In vitro investigation of potential fluorescence contrast agents in 2D MDCK monolayer models

Up to this point the work presented in this thesis has been centred on developing processing algorithms and optical sensors to investigate the potential of transcutaneous fluorescence spectroscopy as a tool to non-invasively assess gut permeability. In this case, Chapter 7 focused on investigating other fluorescence contrast agents that could be used as an alternative to or simultaneously with fluorescein to improve the assessment of gut permeability. Fluorescein has proved to be clinically safe during intravenous or oral administration and has demonstrated to permeate selectively from the gut to the bloodstream under different conditions [90, 148, 151, 186, 188, 206, 209, 234]. Although fluorescein has a similar molecular size as Lactulose, which is widely use in the clinic and in research to assess gut permeability, investigation of complementary fluorescence dyes could provide more information about the permeability in other regions of the intestinal tract. For example, Maurice et al. [90]. showed that fluorescein is mainly absorbed in the small intestine, which limits the assessment of colonic gut dysfunction. In addition, results presented in the previous chapters showed that fluorescein permeated easily in conditions self-categorised as healthy due to, tentatively, its small molecular size. Therefore, the main objective of this chapter was to characterise the permeation of fluorescein within a more controlled environment with different

induced barrier dysfunction levels. Simultaneously, we also investigate the potential of other fluorophores with different molecular size and fluorescence spectra to assess gut permeability. To do so, MDCK monolayer models were used to characterise and compare the permeation of fluorescein with other fluorescence compounds, such as FITC dextran and Methylene Blue. EGTA and Ethanol, at different concentrations, were used to simulate different levels of barrier dysfunction. Although ethanol exhibited a less pronounced and unstable effect, TEER measurements showed that both disruptors compromised the integrity of the tight junctions, leading to increased permeation. Prior to the permeability assays, it was observed that the 0.4 μm inserts filters restricted the permeation of the fluorophores to a maximum of $\approx 4\%$ of the total initial concentration.

Therefore, results from the following experiments were standardised to the maxima achievable for each fluorophore. Considering this, immediate future work should focus on the comparison of insert filters with different pore sizes, providing information about which pore size works best in this case. Some of the potential sizes might include 1 to 3 μm , to prevent MDCK cells to migrate between compartments.

Following from the above observations, the permeation of fluorescein, FITC dextran and Methylene Blue was characterised under different conditions. In the case of Methylene Blue, a potential cell toxicity was detected at concentrations of 1.461 $\mu\text{g}/\text{ml}$ due to a significant decrease in TEER value alongside an increase in the relative permeation. This hypothesis could be comprehensively evaluated in the future using cell viability and functional assays, in which metabolic and mitochondrial function or DNA damage are measured.

In the case of fluorescein and FITC dextran, the results demonstrated that both fluorophores permeated distinctly through the epithelial barrier under different barrier conditions. These differences were mainly attributed to their different molecular sizes, as the passive translocation of molecules, particularly through the paracellular pathway is highly driven by the size of the molecule [3, 12, 14]. More importantly, results from this assay showed that fluorescein exhibited a clearer discrimination between the conditions, suggesting a higher specificity to different levels of epithelial damage than FITC dextran.

As explained previously, the further investigation of the fluorescein permeation was driven by the high fluorescence intensities observed in specific participants in Chapters 5 and 6. However, overall, the findings from this chapter support fluorescein as an effective candidate for assessing gut permeability. Future work should then aim to elucidate the underlying factors contributing to the high fluorescence intensity signals observed in healthy participants. Some potential solutions have been discussed previously and include the investigation of the impact of confounding factors, such as variations in diet, stress levels, alcohol intake or medication, in the gut permeability. Additionally, further work could also centre on investigating and identifying more fluorescent dyes suitable for transcutaneous sensing, including the use of multiple fluorescence dyes to assess permeability in other areas of the intestinal tract. For example, encapsulation of complementary dyes, with a

controlled or delay release of the fluorophores, will open a window to explore different regions of the gut [293, 294].

8.6 Final remarks

Overall, this thesis has focused on the development of processing algorithms and optical sensors to investigate the potential of transcutaneous fluorescence spectroscopy as a tool to non-invasively assess gut permeability. The in vitro and in vivo validation of the devices demonstrated the potential of the technology to measure changes in gut permeability in different conditions and populations. The development of a portable sensor allowed the study of gut permeability in undernourished adults, with promising results showing significant differences between healthy and undernutrition groups. The results further highlighted the limitations of current clinical diagnostic tools, such as sugar permeability tests and biopsies, on the diagnosis of abnormal gut permeability conditions. Further work in this field has been discussed above and the long term aims focus on the development of a low-cost fully wearable transcutaneous fluorescence device that could be used in multiple populations, including children. The device presented in this thesis represents a platform for future investigations towards a better understanding of the mechanisms and interactions behind changes in gut permeability and pathological conditions and to guide the development of better tools for assessing gut barrier function.

References

- [1] M. Vancamelbeke and S. Vermeire. "The intestinal barrier: a fundamental role in health and disease". In: *Expert Rev Gastroenterol Hepatol* 11.9 (2017), pp. 821–834. ISSN: 1747-4132 (Electronic) 1747-4124 (Print) 1747-4124 (Linking). doi: [10.1080/17474124.2017.1343143](https://doi.org/10.1080/17474124.2017.1343143).
- [2] N. Di Tommaso, A. Gasbarrini, and F. R. Ponziani. "Intestinal Barrier in Human Health and Disease". In: *Int J Environ Res Public Health* 18.23 (2021). ISSN: 1660-4601 (Electronic) 1661-7827 (Print) 1660-4601 (Linking). doi: [10.3390/ijerph182312836](https://doi.org/10.3390/ijerph182312836).
- [3] I. Schoultz and A. V. Keita. "The Intestinal Barrier and Current Techniques for the Assessment of Gut Permeability". In: *Cells* 9.8 (2020). ISSN: 2073-4409 (Electronic) 2073-4409 (Linking). doi: [10.3390/cells9081909](https://doi.org/10.3390/cells9081909).
- [4] H. Gehart and H. Clevers. "Tales from the crypt: new insights into intestinal stem cells". In: *Nat Rev Gastroenterol Hepatol* 16.1 (2019), pp. 19–34. ISSN: 1759-5053 (Electronic) 1759-5045 (Linking). doi: [10.1038/s41575-018-0081-y](https://doi.org/10.1038/s41575-018-0081-y).
- [5] O. Kayisoglu, N. Schlegel, and S. Bartfeld. "Gastrointestinal epithelial innate immunity regionalization and organoids as new model". In: *J Mol Med (Berl)* 99.4 (2021), pp. 517–530. ISSN: 1432-1440 (Electronic) 0946-2716 (Print) 0946-2716 (Linking). doi: [10.1007/s00109-021-02043-9](https://doi.org/10.1007/s00109-021-02043-9).
- [6] J. M. Natividad and E. F. Verdu. "Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications". In: *Pharmacol Res* 69.1 (2013), pp. 42–51. ISSN: 1096-1186 (Electronic) 1043-6618 (Linking). doi: [10.1016/j.phrs.2012.10.007](https://doi.org/10.1016/j.phrs.2012.10.007).
- [7] M. Potrykus, M. Szymanski, L. Kaska, and A. Janczy. "Intestinal barrier disorders and metabolic endotoxemia in obesity: Current knowledge". In: *Sciendo* (2022), pp. 71–80. doi: <https://doi.org/10.2478/ahem-2022-0008>.
- [8] S. N. Hong, J. C. Dunn, M. Stelzner, and M. G. Martin. "Concise Review: The Potential Use of Intestinal Stem Cells to Treat Patients with Intestinal Failure". In: *Stem Cells Transl Med* 6.2 (2017), pp. 666–676. ISSN: 2157-6564 (Print) 2157-6580 (Electronic) 2157-6564 (Linking). doi: [10.5966/sctm.2016-0153](https://doi.org/10.5966/sctm.2016-0153).
- [9] S. Kong, Y. H. Zhang, and W. Zhang. "Regulation of Intestinal Epithelial Cells Properties and Functions by Amino Acids". In: *Biomed Res Int* 2018 (2018), p. 2819154. ISSN: 2314-6141 (Electronic) 2314-6133 (Print). doi: [10.1155/2018/2819154](https://doi.org/10.1155/2018/2819154).
- [10] G. Barbara, M. R. Barbaro, D. Fuschi, M. Palombo, F. Falangone, C. Cremon, G. Marasco, and V. Stanghellini. "Inflammatory and Microbiota-Related Regulation of the Intestinal Epithelial Barrier". In: *Front Nutr* 8 (2021), p. 718356. ISSN: 2296-861X (Print) 2296-861X (Electronic) 2296-861X (Linking). doi: [10.3389/fnut.2021.718356](https://doi.org/10.3389/fnut.2021.718356).
- [11] C. Chelakkot, J. Ghim, and S. H. Ryu. "Mechanisms regulating intestinal barrier integrity and its pathological implications". In: *Exp Mol Med* 50.8 (2018), pp. 1–9. ISSN: 2092-6413 (Electronic) 1226-3613 (Print) 1226-3613 (Linking). doi: [10.1038/s12276-018-0126-x](https://doi.org/10.1038/s12276-018-0126-x).
- [12] A. Horowitz, S. D. Chanez-Paredes, X. Haest, and J. R. Turner. "Paracellular permeability and tight junction regulation in gut health and disease". In: *Nat Rev Gastroenterol Hepatol* 20.7 (2023), pp. 417–432. ISSN: 1759-5053 (Electronic) 1759-5045 (Print) 1759-5045 (Linking). doi: [10.1038/s41575-023-00766-3](https://doi.org/10.1038/s41575-023-00766-3).

- [13] B. F. Choonara, Y. E. Choonara, P. Kumar, D. Bijukumar, L. C. du Toit, and V. Pillay. "A review of advanced oral drug delivery technologies facilitating the protection and absorption of protein and peptide molecules". In: *Biotechnol Adv* 32.7 (2014), pp. 1269–1282. ISSN: 1873-1899 (Electronic) 0734-9750 (Linking). doi: [10.1016/j.biotechadv.2014.07.006](https://doi.org/10.1016/j.biotechadv.2014.07.006).
- [14] E. Haroun, P. Kumar, L. Saba, J. Kassab, K. Ghimire, D. Dutta, and S. Lim. "Intestinal barrier functions in hematologic and oncologic diseases". In: *Journal of Translational Medicine* 21 (2023), p. 233. doi: <https://doi.org/10.1186/s12967-023-04091-w>.
- [15] N. Perez-Diaz-Del-Campo, G. Castelnuevo, D. G. Ribaldone, and G. P. Caviglia. "Fecal and Circulating Biomarkers for the Non-Invasive Assessment of Intestinal Permeability". In: *Diagnostics (Basel)* 13.11 (2023). ISSN: 2075-4418 (Print) 2075-4418 (Electronic) 2075-4418 (Linking). doi: [10.3390/diagnostics13111976](https://doi.org/10.3390/diagnostics13111976).
- [16] C. T. Okamoto. "Endocytosis and transcytosis". In: *Adv Drug Deliv Rev* 29.3 (1998), pp. 215–228. ISSN: 1872-8294 (Electronic) 0169-409X (Linking). doi: [10.1016/s0169-409x\(97\)00080-x](https://doi.org/10.1016/s0169-409x(97)00080-x).
- [17] B. Meijers, R. Farre, S. Dejongh, M. Vicario, and P. Evenepoel. "Intestinal Barrier Function in Chronic Kidney Disease". In: *Toxins (Basel)* 10.7 (2018). ISSN: 2072-6651 (Electronic) 2072-6651 (Linking). doi: [10.3390/toxins10070298](https://doi.org/10.3390/toxins10070298).
- [18] T. Suzuki. "Regulation of intestinal epithelial permeability by tight junctions". In: *Cell Mol Life Sci* 70.4 (2013), pp. 631–59. ISSN: 1420-9071 (Electronic) 1420-682X (Linking). doi: [10.1007/s00018-012-1070-x](https://doi.org/10.1007/s00018-012-1070-x).
- [19] A. Ali, H. Tan, and G. E. Kaiko. "Role of the Intestinal Epithelium and Its Interaction With the Microbiota in Food Allergy". In: *Front Immunol* 11 (2020), p. 604054. ISSN: 1664-3224 (Electronic) 1664-3224 (Linking). doi: [10.3389/fimmu.2020.604054](https://doi.org/10.3389/fimmu.2020.604054).
- [20] J. M. Anderson and C. M. Van Itallie. "Physiology and function of the tight junction". In: *Cold Spring Harb Perspect Biol* 1.2 (2009), a002584. ISSN: 1943-0264 (Electronic) 1943-0264 (Linking). doi: [10.1101/cshperspect.a002584](https://doi.org/10.1101/cshperspect.a002584).
- [21] M. Cereijido, J. Valdes, L. Shoshani, and R. G. Contreras. "Role of tight junctions in establishing and maintaining cell polarity". In: *Annu Rev Physiol* 60 (1998), pp. 161–77. ISSN: 0066-4278 (Print) 0066-4278 (Linking). doi: [10.1146/annurev.physiol.60.1.161](https://doi.org/10.1146/annurev.physiol.60.1.161).
- [22] M. Balda and K. Matter. "Tight junctions at a glance". In: *Cel Science* 121.22 (2008), pp. 3677–3682. doi: <https://doi.org/10.1242/jcs.023887>.
- [23] L. Zuo, W. T. Kuo, and J. R. Turner. "Tight Junctions as Targets and Effectors of Mucosal Immune Homeostasis". In: *Cell Mol Gastroenterol Hepatol* 10 (2020), pp. 327–340. ISSN: 2352-345x. doi: [10.1016/j.jcmgh.2020.04.001](https://doi.org/10.1016/j.jcmgh.2020.04.001).
- [24] C. T. Capaldo and A. Nusrat. "Cytokine regulation of tight junctions". In: *Biochim Biophys Acta* 1788.4 (2009), pp. 864–71. ISSN: 0006-3002 (Print) 0006-3002 (Linking). doi: [10.1016/j.bbamem.2008.08.027](https://doi.org/10.1016/j.bbamem.2008.08.027).
- [25] M. A. Odenwald and J. R. Turner. "The intestinal epithelial barrier: a therapeutic target?" In: *Nat Rev Gastroenterol Hepatol* 14.1 (2017), pp. 9–21. ISSN: 1759-5053 (Electronic) 1759-5045 (Print) 1759-5045 (Linking). doi: [10.1038/nrgastro.2016.169](https://doi.org/10.1038/nrgastro.2016.169).
- [26] M. A. Odenwald and J. R. Turner. "Intestinal permeability defects: is it time to treat?" In: *Clin Gastroenterol Hepatol* 11.9 (2013), pp. 1075–83. ISSN: 1542-7714 (Electronic) 1542-3565 (Print) 1542-3565 (Linking). doi: [10.1016/j.cgh.2013.07.001](https://doi.org/10.1016/j.cgh.2013.07.001).
- [27] C. Graziani, C. Talocco, R. De Sire, et al. "Intestinal permeability in physiological and pathological conditions: major determinants and assessment modalities". In: *Eur Rev Med Pharmacol Sci* 23.2 (2019), pp. 795–810. ISSN: 2284-0729 (Electronic) 1128-3602 (Linking). doi: [10.26355/eurev.201901_16894](https://doi.org/10.26355/eurev.201901_16894).

- [28] V. Owino, T. Ahmed, M. Freemark, P. Kelly, A. Loy, M. Manary, and C. Loechl. "Environmental Enteric Dysfunction and Growth Failure/Stunting in Global Child Health". In: *Pediatrics* 138.6 (2016). issn: 1098-4275 (Electronic) 0031-4005 (Linking). doi: [10.1542/peds.2016-0641](https://doi.org/10.1542/peds.2016-0641).
- [29] C. Skinner, A. J. Thompson, M. R. Thursz, J. R. Marchesi, and N. Vergis. "Intestinal permeability and bacterial translocation in patients with liver disease, focusing on alcoholic aetiology: methods of assessment and therapeutic intervention". In: *Therap Adv Gastroenterol* 13 (2020), p. 1756284820942616. issn: 1756-283X (Print) 1756-2848 (Electronic) 1756-283X (Linking). doi: [10.1177/1756284820942616](https://doi.org/10.1177/1756284820942616).
- [30] L. V. Hooper, D. R. Littman, and A. J. Macpherson. "Interactions between the microbiota and the immune system". In: *Science* 336.6086 (2012), pp. 1268–73. issn: 1095-9203 (Electronic) 0036-8075 (Print) 0036-8075 (Linking). doi: [10.1126/science.1223490](https://doi.org/10.1126/science.1223490).
- [31] A. Buckley and J. R. Turner. "Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease". In: *Cold Spring Harb Perspect Biol* 10.1 (2018). issn: 1943-0264 (Electronic) 1943-0264 (Linking). doi: [10.1101/cshperspect.a029314](https://doi.org/10.1101/cshperspect.a029314).
- [32] S. C. Nalle, H. A. Kwak, K. L. Edelblum, N. E. Joseph, G. Singh, G. F. Khramtsova, E. D. Mortenson, P. A. Savage, and J. R. Turner. "Recipient NK cell inactivation and intestinal barrier loss are required for MHC-matched graft-versus-host disease". In: *Sci Transl Med* 6.243 (2014), 243ra87. issn: 1946-6242 (Electronic) 1946-6234 (Print) 1946-6234 (Linking). doi: [10.1126/scitranslmed.3008941](https://doi.org/10.1126/scitranslmed.3008941).
- [33] F. Liu, S. A. Lee, S. M. Riordan, L. Zhang, and L. Zhu. "Effects of Anti-Cytokine Antibodies on Gut Barrier Function". In: *Mediators Inflamm* 2019 (2019), p. 7028253. issn: 1466-1861 (Electronic) 0962-9351 (Print) 0962-9351 (Linking). doi: [10.1155/2019/7028253](https://doi.org/10.1155/2019/7028253).
- [34] F. Meyer, D. Wendling, C. Demougeot, C. Prati, and F. Verhoeven. "Cytokines and intestinal epithelial permeability: A systematic review". In: *Autoimmun Rev* 22.6 (2023), p. 103331. issn: 1873-0183 (Electronic) 1568-9972 (Linking). doi: [10.1016/j.autrev.2023.103331](https://doi.org/10.1016/j.autrev.2023.103331).
- [35] J. D. Soderholm, G. Olaison, E. Lindberg, U. Hannestad, A. Vindels, C. Tysk, G. Järnerot, and R. Sjö Dahl. "Different intestinal permeability patterns in relatives and spouses of patients with Crohn's disease: an inherited defect in mucosal defence?" In: *Gut* 44.1 (1999), pp. 96–100. issn: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.44.1.96](https://doi.org/10.1136/gut.44.1.96).
- [36] A. Thompson, C. Bourke, R. Robertson, et al. "Understanding the role of the gut in undernutrition: what can technology tell us?" In: *Gut* 70 (2021), pp. 1580–94. doi: <https://doi.org/10.1136/gutjnl-2020-323609>.
- [37] R. D'Inca, V. Di Leo, G. Corrao, D. Martines, A. D'Odorico, C. Mestriner, C. Venturi, G. Longo, and G. C. Sturniolo. "Intestinal permeability test as a predictor of clinical course in Crohn's disease". In: *Am J Gastroenterol* 94.10 (1999), pp. 2956–60. issn: 0002-9270 (Print) 0002-9270 (Linking). doi: [10.1111/j.1572-0241.1999.01444.x](https://doi.org/10.1111/j.1572-0241.1999.01444.x).
- [38] J. Wyatt, H. Vogelsang, W. Hubl, T. Waldhoer, and H. Lochs. "Intestinal permeability and the prediction of relapse in Crohn's disease". In: *Lancet* 341.8858 (1993), pp. 1437–9. issn: 0140-6736. doi: [10.1016/0140-6736\(93\)90882-h](https://doi.org/10.1016/0140-6736(93)90882-h).
- [39] J. A. Tibble, G. Sigthorsson, S. Bridger, M. K. Fagerhol, and I. Bjarnason. "Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease". In: *Gastroenterology* 119.1 (2000), pp. 15–22. issn: 0016-5085 (Print) 0016-5085 (Linking). doi: [10.1053/gast.2000.8523](https://doi.org/10.1053/gast.2000.8523).
- [40] P. Kohout. "Small bowel permeability in diagnosis of celiac disease and monitoring of compliance of a gluten-free diet (gut permeability in celiac disease)". In: *Acta Medica (Hradec Kralove)* 44.3 (2001), pp. 101–4. issn: 1211-4286 (Print) 1211-4286 (Linking).

- [41] E. Smecuol, J. C. Bai, H. Vazquez, Z. Kogan, A. Cabanne, S. Niveloni, S. Pedreira, L. Boerr, E. Maurino, and J. B. Meddings. "Gastrointestinal permeability in celiac disease". In: *Gastroenterology* 112.4 (1997), pp. 1129–36. ISSN: 0016-5085 (Print) 0016-5085 (Linking). doi: [10.1016/s0016-5085\(97\)70123-9](https://doi.org/10.1016/s0016-5085(97)70123-9).
- [42] J. Visser, J. Rozing, A. Sapone, K. Lammers, and A. Fasano. "Tight junctions, intestinal permeability, and autoimmunity: celiac disease and type 1 diabetes paradigms". In: *Ann N Y Acad Sci* 1165 (2009), pp. 195–205. ISSN: 1749-6632 (Electronic) 0077-8923 (Print) 0077-8923 (Linking). doi: [10.1111/j.1749-6632.2009.04037.x](https://doi.org/10.1111/j.1749-6632.2009.04037.x).
- [43] D. R. Duerksen, C. Wilhelm-Boyles, and D. M. Parry. "Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet". In: *Dig Dis Sci* 50.4 (2005), pp. 785–90. ISSN: 0163-2116 (Print) 0163-2116 (Linking). doi: [10.1007/s10620-005-2574-0](https://doi.org/10.1007/s10620-005-2574-0).
- [44] I. S. Menzies, M. J. Zuckerman, W. S. Nukajam, S. G. Somasundaram, B. Murphy, A. P. Jenkins, R. S. Crane, and G. G. Gregory. "Geography of intestinal permeability and absorption". In: *Gut* 44.4 (1999), pp. 483–9. ISSN: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.44.4.483](https://doi.org/10.1136/gut.44.4.483).
- [45] A. K. Dutta and A. Chacko. "Influence of environmental factors on the onset and course of inflammatory bowel disease". In: *World J Gastroenterol* 22.3 (2016), pp. 1088–100. ISSN: 2219-2840 (Electronic) 1007-9327 (Print) 1007-9327 (Linking). doi: [10.3748/wjg.v22.i3.1088](https://doi.org/10.3748/wjg.v22.i3.1088).
- [46] C. Siew, C. Bernstein, M. Vatn, P. Lakatos, E. Loftus Jr, C. Tysk, C. O'Marain, B. Moum, and J. Colombel. "Geographical variability and environmental risk factors in inflammatory bowel disease". In: *Gut* 62 (2013), pp. 630–649. doi: [10.1136/gutjnl-2012-303661](https://doi.org/10.1136/gutjnl-2012-303661).
- [47] L. Biedermann and G. Rogler. "Environmental factors and their impact on the intestinal microbiota: a role for human disease?" In: *Digestive Disease* 30 (2013), pp. 20–27. doi: <https://doi.org/10.1159/000342590>.
- [48] I. Logan and C. Bowlus. "The geoepidemiology of autoimmune intestinal diseases". In: *Autoimmun Rev* 9.5 (2010), A372–378. doi: <https://doi.org/10.1016/j.autrev.2009.11.008>.
- [49] C. Chapman-Kiddell, S. Davies, L. Gillen, and G. Radford-Smith. "Role of diet in the development of inflammatory bowel disease". In: *Inflammatory Bowel Diseases* 16.1 (2010), pp. 137–151. doi: <https://doi.org/10.1002/ibd.20968>.
- [50] J. W. Windsor and G. G. Kaplan. "Evolving Epidemiology of IBD". In: *Curr Gastroenterol Rep* 21.8 (2019), p. 40. ISSN: 1534-312X. doi: [10.1007/s11894-019-0705-6](https://doi.org/10.1007/s11894-019-0705-6).
- [51] S. M. Fletcher, M. L. McLaws, and J. T. Ellis. "Prevalence of gastrointestinal pathogens in developed and developing countries: systematic review and meta-analysis". In: *J Public Health Res* 2.1 (2013), pp. 42–53. ISSN: 2279-9028 (Print) 2279-9036 (Electronic) 2279-9028 (Linking). doi: [10.4081/jphr.2013.e9](https://doi.org/10.4081/jphr.2013.e9).
- [52] R. K. Campbell, K. J. Schulze, S. Shaikh, et al. "Biomarkers of Environmental Enteric Dysfunction Among Children in Rural Bangladesh". In: *J Pediatr Gastroenterol Nutr* 65.1 (2017), pp. 40–46. ISSN: 1536-4801 (Electronic) 0277-2116 (Print) 0277-2116 (Linking). doi: [10.1097/MPG.0000000000001557](https://doi.org/10.1097/MPG.0000000000001557).
- [53] R. Crane, K. Jones, and J. Berkley. "Environmental enteric dysfunction: an overview". In: *Food and Nutrition Bulletin* 36.1 (2015), S3–S92. doi: doi.org/10.1177/15648265150361S113.
- [54] M. Batista de Moraes and G. Pontes da Silva. "Environmental enteric dysfunction and growth". In: *Pediatrics* 95 (2019), S85–S94. doi: <https://doi.org/10.1016/j.jpeds.2018.11.004>.
- [55] G. C. Cook, S. K. Kajubi, and F. D. Lee. "Jejunal morphology of the African in Uganda". In: *J Pathol* 98.3 (1969), pp. 157–69. ISSN: 0022-3417 (Print) 0022-3417 (Linking). doi: [10.1002/path.1710980302](https://doi.org/10.1002/path.1710980302).

- [56] P. Hodges, M. Tembo, and P. Kelly. "Intestinal Biopsies for the Evaluation of Environmental Enteropathy and Environmental Enteric Dysfunction". In: *J Infect Dis* 224.12 Suppl 2 (2021), S856–S863. ISSN: 1537-6613 (Electronic) 0022-1899 (Print) 0022-1899 (Linking). doi: [10.1093/infdis/jiab372](https://doi.org/10.1093/infdis/jiab372).
- [57] G. T. Keusch, D. M. Denno, R. E. Black, et al. "Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences". In: *Clin Infect Dis* 59 Suppl 4. Suppl 4 (2014), S207–12. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). doi: [10.1093/cid/ciu485](https://doi.org/10.1093/cid/ciu485).
- [58] F. K. Welsh, S. M. Farmery, K. MacLennan, M. B. Sheridan, G. R. Barclay, P. J. Guillou, and J. V. Reynolds. "Gut barrier function in malnourished patients". In: *Gut* 42.3 (1998), pp. 396–401. ISSN: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.42.3.396](https://doi.org/10.1136/gut.42.3.396).
- [59] J. Petri W. A., C. Naylor, and R. Haque. "Environmental enteropathy and malnutrition: do we know enough to intervene?" In: *BMC Med* 12 (2014), p. 187. ISSN: 1741-7015 (Electronic) 1741-7015 (Linking). doi: [10.1186/s12916-014-0187-1](https://doi.org/10.1186/s12916-014-0187-1).
- [60] S. McKay, E. Gaudier, D. I. Campbell, A. M. Prentice, and R. Albers. "Environmental enteropathy: new targets for nutritional interventions". In: *Int Health* 2.3 (2010), pp. 172–80. ISSN: 1876-3413 (Print) 1876-3405 (Linking). doi: [10.1016/j.inhe.2010.07.006](https://doi.org/10.1016/j.inhe.2010.07.006).
- [61] World Health Organization. *Malnutrition*. Web Page.
- [62] D. A. Fufa and T. D. Laloto. "Factors associated with undernutrition among children aged between 6-36 months in Semien Bench district, Ethiopia". In: *Heliyon* 7.5 (2021), e07072. ISSN: 2405-8440 (Print) 2405-8440 (Electronic) 2405-8440 (Linking). doi: [10.1016/j.heliyon.2021.e07072](https://doi.org/10.1016/j.heliyon.2021.e07072).
- [63] K. G. Dewey and K. Begum. "Long-term consequences of stunting in early life". In: *Matern Child Nutr* 7 Suppl 3. Suppl 3 (2011), pp. 5–18. ISSN: 1740-8709 (Electronic) 1740-8695 (Print) 1740-8695 (Linking). doi: [10.1111/j.1740-8709.2011.00349.x](https://doi.org/10.1111/j.1740-8709.2011.00349.x).
- [64] R. E. Black, L. H. Allen, Z. A. Bhutta, L. E. Caulfield, M. de Onis, M. Ezzati, C. Mathers, J. Rivera, Maternal, and G. Child Undernutrition Study. "Maternal and child undernutrition: global and regional exposures and health consequences". In: *Lancet* 371.9608 (2008), pp. 243–260. ISSN: 1474-547X (Electronic) 0140-6736 (Linking). doi: [10.1016/S0140-6736\(07\)61690-0](https://doi.org/10.1016/S0140-6736(07)61690-0).
- [65] A. K. Kiani, K. Dhuli, K. Donato, et al. "Main nutritional deficiencies". In: *J Prev Med Hyg* 63.2 Suppl 3 (2022), E93–E101. ISSN: 2421-4248 (Electronic) 1121-2233 (Print) 1121-2233 (Linking). doi: [10.15167/2421-4248/jpmh2022.63.2S3.2752](https://doi.org/10.15167/2421-4248/jpmh2022.63.2S3.2752).
- [66] K. Maleta. "Undernutrition". In: *Malawi Med J* 18.4 (2006), pp. 189–205. ISSN: 1995-7262 (Print) 1995-7270 (Electronic) 1995-7262 (Linking).
- [67] Z. Grover and L. C. Ee. "Protein energy malnutrition". In: *Pediatr Clin North Am* 56.5 (2009), pp. 1055–68. ISSN: 1557-8240 (Electronic) 0031-3955 (Linking). doi: [10.1016/j.pcl.2009.07.001](https://doi.org/10.1016/j.pcl.2009.07.001).
- [68] M. Rahman. "Association between malnutrition and anemia in under-five children and women of reproductive age: Evidence from Bangladesh Demographic and Health Survey 2011". In: *PLoS One* 14.7 (2019), e0219170. doi: <https://doi.org/10.1371/journal.pone.0219170>.
- [69] C. Hodge and C. Taylor. "Vitamin A Deficiency". In: *StatPearls*. Treasure Island (FL), 2024.
- [70] P. Webb, G. A. Stordalen, S. Singh, R. Wijesinha-Bettoni, P. Shetty, and A. Lartey. "Hunger and malnutrition in the 21st century". In: *BMJ* 361 (2018), k2238. ISSN: 1756-1833 (Electronic) 0959-8138 (Print) 0959-8138 (Linking). doi: [10.1136/bmj.k2238](https://doi.org/10.1136/bmj.k2238).

- [71] A. M. Williams and P. S. Suchdev. "Assessing and Improving Childhood Nutrition and Growth Globally". In: *Pediatr Clin North Am* 64.4 (2017), pp. 755–768. ISSN: 1557-8240. doi: [10.1016/j.pcl.2017.03.001](https://doi.org/10.1016/j.pcl.2017.03.001).
- [72] W. World Health Organization. *The UNICEF/WHO/WB Joint Child Malnutrition Estimates (JME) group released new data*. Press Release. 2020.
- [73] Department of International Development, (DFID) and UKaid. *The neglected crisis of under-nutrition: evidence fro action*. Report. Department for international development, 2008.
- [74] (UNSCN), Initiatives Development and United Nations System Standing Committee on Nutrition. *2018 Global Nutrition Report: shinning a light to spur action on nutrition*. Report. 2018.
- [75] E. Tzioumis, M. C. Kay, M. E. Bentley, and L. S. Adair. "Prevalence and trends in the childhood dual burden of malnutrition in low- and middle-income countries, 1990-2012". In: *Public Health Nutr* 19.8 (2016), pp. 1375–88. ISSN: 1475-2727 (Electronic) 1368-9800 (Print) 1368-9800 (Linking). doi: [10.1017/S1368980016000276](https://doi.org/10.1017/S1368980016000276).
- [76] G. Global Health Observatory. *Dataset - Underweight among children under 5 years of age*. Online Database. Mar. 2024.
- [77] H. Delisle, M. Faber, and P. Revault. "Evidence-based strategies needed to combat malnutrition in Sub-Saharan countries facing different stages of nutrition transition". In: *Public Health Nutr* 24.12 (2021), pp. 3577–3580. ISSN: 1475-2727 (Electronic) 1368-9800 (Print) 1368-9800 (Linking). doi: [10.1017/S1368980021001221](https://doi.org/10.1017/S1368980021001221).
- [78] F. Ramponi, W. Tafesse, and S. Griffin. "Economic evaluation of interventions to address undernutrition: a systematic review". In: *Health Policy Plan* 36.4 (2021), pp. 533–541. ISSN: 1460-2237 (Electronic) 0268-1080 (Print) 0268-1080 (Linking). doi: [10.1093/heapol/czaa149](https://doi.org/10.1093/heapol/czaa149).
- [79] S. B. Soofi, G. N. Khan, S. Ariff, Y. Ihtesham, M. Tanimoune, A. Rizvi, M. Sajid, C. Garzon, S. de Pee, and Z. A. Bhutta. "Effectiveness of nutritional supplementation during the first 1000-days of life to reduce child undernutrition: A cluster randomized controlled trial in Pakistan". In: *Lancet Reg Health Southeast Asia* 4 (2022), p. 100035. ISSN: 2772-3682 (Electronic) 2772-3682 (Linking). doi: [10.1016/j.lansea.2022.100035](https://doi.org/10.1016/j.lansea.2022.100035).
- [80] S. P. Luby, M. Rahman, B. F. Arnold, et al. "Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Bangladesh: a cluster randomised controlled trial". In: *Lancet Glob Health* 6.3 (2018), e302–e315. ISSN: 2214-109X (Electronic) 2214-109X (Linking). doi: [10.1016/S2214-109X\(17\)30490-4](https://doi.org/10.1016/S2214-109X(17)30490-4).
- [81] C. Null, C. P. Stewart, A. J. Pickering, et al. "Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Kenya: a cluster-randomised controlled trial". In: *Lancet Glob Health* 6.3 (2018), e316–e329. ISSN: 2214-109X (Electronic) 2214-109X (Linking). doi: [10.1016/S2214-109X\(18\)30005-6](https://doi.org/10.1016/S2214-109X(18)30005-6).
- [82] J. H. Humphrey, M. N. N. Mbuya, R. Ntozini, et al. "Independent and combined effects of improved water, sanitation, and hygiene, and improved complementary feeding, on child stunting and anaemia in rural Zimbabwe: a cluster-randomised trial". In: *Lancet Glob Health* 7.1 (2019), e132–e147. ISSN: 2214-109X (Electronic) 2214-109X (Linking). doi: [10.1016/S2214-109X\(18\)30374-7](https://doi.org/10.1016/S2214-109X(18)30374-7).
- [83] A. J. Prendergast, B. Chasekwa, C. Evans, et al. "Independent and combined effects of improved water, sanitation, and hygiene, and improved complementary feeding, on stunting and anaemia among HIV-exposed children in rural Zimbabwe: a cluster-randomised controlled trial". In: *Lancet Child Adolesc Health* 3.2 (2019), pp. 77–90. ISSN: 2352-4650 (Electronic) 2352-4642 (Print) 2352-4642 (Linking). doi: [10.1016/S2352-4642\(18\)30340-7](https://doi.org/10.1016/S2352-4642(18)30340-7).
- [84] L. E. Smith, D. Chagwena, C. Bourke, et al. "Child Health, Agriculture and integrated nutrition (CHAIN): protocol for a randomised controlled trial of improved infant and young child

- feeding in rural Zimbabwe." In: *BMJ Open* 12.e056435 (2022). doi: [10.1136/bmjopen-2021-056435](https://doi.org/10.1136/bmjopen-2021-056435).
- [85] G. Kathryn and S. A. "Systematic review of the efficacy and effectiveness of complementary feeding interventions in developing countries". In: *Maternal and Child Nutrition* (2008). doi: <https://doi.org/10.1111/j.1740-8709.2007.00124.x>.
- [86] T. Vanuytsel, J. Tack, and R. Farre. "The Role of Intestinal Permeability in Gastrointestinal Disorders and Current Methods of Evaluation". In: *Front Nutr* 8 (2021), p. 717925. ISSN: 2296-861X (Print) 2296-861X (Electronic) 2296-861X (Linking). doi: [10.3389/fnut.2021.717925](https://doi.org/10.3389/fnut.2021.717925).
- [87] A. Fasano. "All disease begins in the (leaky) gut: role of zonulin-mediated gut permeability in the pathogenesis of some chronic inflammatory diseases". In: *F1000Research* 9.69 (2020). doi: <https://doi.org/10.12688/f1000research.20510.1>.
- [88] H. J. Galipeau and E. F. Verdu. "The complex task of measuring intestinal permeability in basic and clinical science". In: *Neurogastroenterol Motil* 28.7 (2016), pp. 957–65. ISSN: 1365-2982 (Electronic) 1350-1925 (Linking). doi: [10.1111/nmo.12871](https://doi.org/10.1111/nmo.12871).
- [89] A. Thompson, M. Hughes, S. Anastasova, et al. "The potential role of optical biopsy in the study an diagnosis of environmental enteric dysfunction". In: *Nature Reviews Gastroenterology and Hepatology* 14 (2017), pp. 727–38. doi: <https://doi.org/10.1038/nrgastro.2017.147>.
- [90] J. Maurice, A. M. Lett, C. Skinner, et al. "Transcutaneous fluorescence spectroscopy as a tool for non-invasive monitoring of gut function: first clinical experiences". In: *Science Reports* 10 (2020), p. 16169. doi: <https://doi.org/10.1038/s41598-020-73149-2>.
- [91] T. Rath, R. Atreya, J. Bodenschatz, et al. "Intestinal Barrier Healing Is Superior to Endoscopic and Histologic Remission for Predicting Major Adverse Outcomes in Inflammatory Bowel Disease: The Prospective ERLca Trial". In: *Gastroenterology* 164.2 (2023), pp. 241–255. ISSN: 1528-0012 (Electronic) 0016-5085 (Linking). doi: [10.1053/j.gastro.2022.10.014](https://doi.org/10.1053/j.gastro.2022.10.014).
- [92] T. Rath, W. Dieterich, C. Katscher-Murad, M. F. Neurath, and Y. Zopf. "Cross-sectional imaging of intestinal barrier dysfunction by confocal laser endomicroscopy can identify patients with food allergy in vivo with high sensitivity". In: *Sci Rep* 11.1 (2021), p. 12777. ISSN: 2045-2322 (Electronic) 2045-2322 (Linking). doi: [10.1038/s41598-021-92262-4](https://doi.org/10.1038/s41598-021-92262-4).
- [93] A. Peixoto, M. Silva, P. Pereira, and G. Macedo. "Biopsies in Gastrointestinal Endoscopy: When and How". In: *GE Port J Gastroenterol* 23.1 (2016), pp. 19–27. ISSN: 2341-4545 (Print) 2387-1954 (Electronic) 2387-1954 (Linking). doi: [10.1016/j.jpge.2015.07.004](https://doi.org/10.1016/j.jpge.2015.07.004).
- [94] R. Fiocca and P. Ceppa. "Endoscopic biopsies". In: *J Clin Pathol* 56.5 (2003), pp. 321–2. ISSN: 0021-9746 (Print) 1472-4146 (Electronic) 0021-9746 (Linking). doi: [10.1136/jcp.56.5.321](https://doi.org/10.1136/jcp.56.5.321).
- [95] S. Bharadwaj, N. Narula, P. Tandon, and M. Yaghoobi. "Role of endoscopy in inflammatory bowel disease". In: *Gastroenterol Rep (Oxf)* 6.2 (2018), pp. 75–82. ISSN: 2052-0034 (Print) 2052-0034 (Electronic). doi: [10.1093/gastro/goy006](https://doi.org/10.1093/gastro/goy006).
- [96] M. S. Hossain, S. Begum, M. M. Rahman, et al. "Alterations in the histological features of the intestinal mucosa in malnourished adults of Bangladesh". In: *Sci Rep* 11.1 (2021), p. 2355. ISSN: 2045-2322 (Electronic) 2045-2322 (Linking). doi: [10.1038/s41598-021-82079-6](https://doi.org/10.1038/s41598-021-82079-6).
- [97] G. Rastogi and S. Chawhan. "Histopathology of endoscopic biopsies of gastrointestinal tract lesions with clinical correlation". In: *Acta Medica International* 10.2 (2023), pp. 79–90. doi: [10.4103/amit.amit.8_23](https://doi.org/10.4103/amit.amit.8_23).
- [98] S. Syed, S. Yeruva, J. Herrmann, et al. "Environmental Enteropathy in Undernourished Pakistani Children: Clinical and Histomorphometric Analyses". In: *Am J Trop Med Hyg* 98.6 (2018), pp. 1577–1584. ISSN: 1476-1645 (Electronic) 0002-9637 (Print) 0002-9637 (Linking). doi: [10.4269/ajtmh.17-0306](https://doi.org/10.4269/ajtmh.17-0306).
- [99] S. Syed and S. R. Moore. "Tissue is the Issue: Duodenal Biopsies to Elucidate Gut Structure and Function Among Undernourished Children in Low-Resource Settings". In: *EBioMedicine*

- 23 (2017), pp. 10–11. ISSN: 2352-3964 (Electronic) 2352-3964 (Linking). doi: [10.1016/j.ebiom.2017.08.003](https://doi.org/10.1016/j.ebiom.2017.08.003).
- [100] Z. Alshamy, K. C. Richardson, H. Hunigen, H. M. Hafez, J. Plendl, and S. Al Masri. "Comparison of the gastrointestinal tract of a dual-purpose to a broiler chicken line: A qualitative and quantitative macroscopic and microscopic study". In: *PLoS One* 13.10 (2018), e0204921. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). doi: [10.1371/journal.pone.0204921](https://doi.org/10.1371/journal.pone.0204921).
- [101] R. K. Pai. "A practical approach to small bowel biopsy interpretation: celiac disease and its mimics". In: *Semin Diagn Pathol* 31.2 (2014), pp. 124–36. ISSN: 0740-2570 (Print) 0740-2570 (Linking). doi: [10.1053/j.semdp.2014.02.006](https://doi.org/10.1053/j.semdp.2014.02.006).
- [102] W. M. Wong and N. A. Wright. "Cell proliferation in gastrointestinal mucosa". In: *J Clin Pathol* 52.5 (1999), pp. 321–33. ISSN: 0021-9746. doi: [10.1136/jcp.52.5.321](https://doi.org/10.1136/jcp.52.5.321).
- [103] L. A. Denson. "Application of mucosal functional genomics to childhood undernutrition and stunting: Insights into mechanisms and targeted interventions". In: *EBioMedicine* 71 (2021), p. 103553. ISSN: 2352-3964 (Electronic) 2352-3964 (Linking). doi: [10.1016/j.ebiom.2021.103553](https://doi.org/10.1016/j.ebiom.2021.103553).
- [104] N. T. Iqbal, S. Syed, K. Sadiq, et al. "Study of Environmental Enteropathy and Malnutrition (SEEM) in Pakistan: protocols for biopsy based biomarker discovery and validation". In: *BMC Pediatr* 19.1 (2019), p. 247. ISSN: 1471-2431 (Electronic) 1471-2431 (Linking). doi: [10.1186/s12887-019-1564-x](https://doi.org/10.1186/s12887-019-1564-x).
- [105] A. Georgiadis and M. E. Penny. "Child undernutrition: opportunities beyond the first 1000 days". In: *Lancet Public Health* 2.9 (2017), e399. ISSN: 2468-2667 (Electronic). doi: [10.1016/S2468-2667\(17\)30154-8](https://doi.org/10.1016/S2468-2667(17)30154-8).
- [106] G. T. Keusch, I. H. Rosenberg, D. M. Denno, et al. "Implications of acquired environmental enteric dysfunction for growth and stunting in infants and children living in low- and middle-income countries". In: *Food Nutr Bull* 34.3 (2013), pp. 357–64. ISSN: 0379-5721 (Print) 1564-8265 (Electronic) 0379-5721 (Linking). doi: [10.1177/156482651303400308](https://doi.org/10.1177/156482651303400308).
- [107] I. Levy and I. M. Gralnek. "Complications of diagnostic colonoscopy, upper endoscopy, and enteroscopy". In: *Best Pract Res Clin Gastroenterol* 30.5 (2016), pp. 705–718. ISSN: 1532-1916 (Electronic) 1521-6918 (Linking). doi: [10.1016/j.bpg.2016.09.005](https://doi.org/10.1016/j.bpg.2016.09.005).
- [108] M. D. Yao, E. C. von Rosenvinge, C. Groden, and P. J. Mannon. "Multiple endoscopic biopsies in research subjects: safety results from a National Institutes of Health series". In: *Gastrointest Endosc* 69.4 (2009), pp. 906–10. ISSN: 1097-6779. doi: [10.1016/j.gie.2008.05.015](https://doi.org/10.1016/j.gie.2008.05.015).
- [109] K. M. Harper, M. Mutasa, A. J. Prendergast, J. Humphrey, and A. R. Manges. "Environmental enteric dysfunction pathways and child stunting: A systematic review". In: *PLoS Negl Trop Dis* 12.1 (2018), e0006205. ISSN: 1935-2735 (Electronic) 1935-2727 (Print) 1935-2727 (Linking). doi: [10.1371/journal.pntd.0006205](https://doi.org/10.1371/journal.pntd.0006205).
- [110] M. Samer Ammar, M. D. Pfefferkorn, J. M. Croffie, S. K. Gupta, M. R. Corkins, and J. F. Fitzgerald. "Complications after outpatient upper GI endoscopy in children: 30-day follow-up". In: *Am J Gastroenterol* 98.7 (2003), pp. 1508–11. ISSN: 0002-9270 (Print) 0002-9270 (Linking). doi: [10.1111/j.1572-0241.2003.07524.x](https://doi.org/10.1111/j.1572-0241.2003.07524.x).
- [111] T. M. Attard, A. M. Grima, and M. Thomson. "Pediatric Endoscopic Procedure Complications". In: *Curr Gastroenterol Rep* 20.10 (2018), p. 48. ISSN: 1534-312X (Electronic) 1522-8037 (Linking). doi: [10.1007/s11894-018-0646-5](https://doi.org/10.1007/s11894-018-0646-5).
- [112] Y. J. Kim. "General considerations and updates in pediatric gastrointestinal diagnostic endoscopy". In: *Korean J Pediatr* 53.9 (2010), pp. 817–23. ISSN: 2092-7258 (Electronic) 1738-1061 (Print) 1738-1061 (Linking). doi: [10.3345/kjp.2010.53.9.817](https://doi.org/10.3345/kjp.2010.53.9.817).
- [113] A. Basil and W. Wassef. *Confocal Endomicroscopy - Principles and applications in Medicine, Biology and the Food Sciences*. InTech, 2012. doi: [10.5772/55959](https://doi.org/10.5772/55959).

- [114] S. Chiriac, C. V. Sfarti, H. Minea, et al. "Impaired Intestinal Permeability Assessed by Confocal Laser Endomicroscopy-A New Potential Therapeutic Target in Inflammatory Bowel Disease". In: *Diagnostics (Basel)* 13.7 (2023). ISSN: 2075-4418 (Print) 2075-4418 (Electronic) 2075-4418 (Linking). doi: [10.3390/diagnostics13071230](https://doi.org/10.3390/diagnostics13071230).
- [115] M. Rusticeanu, V. Zimmer, and F. Lammert. "Visualising and quantifying intestinal permeability -where do we stand". In: *Ann Hepatol* 23 (2021), p. 100266. ISSN: 1665-2681 (Print) 1665-2681 (Linking). doi: [10.1016/j.aohp.2020.09.010](https://doi.org/10.1016/j.aohp.2020.09.010).
- [116] M. Goetz, A. Watson, and R. Kiesslich. "Confocal laser endomicroscopy in gastrointestinal diseases". In: *J Biophotonics* 4.7-8 (2011), pp. 498–508. ISSN: 1864-0648 (Electronic) 1864-063X (Linking). doi: [10.1002/jbio.201100022](https://doi.org/10.1002/jbio.201100022).
- [117] M. I. Hossain, B. Nahar, J. D. Hamadani, T. Ahmed, A. K. Roy, and K. H. Brown. "Intestinal mucosal permeability of severely underweight and nonmalnourished Bangladeshi children and effects of nutritional rehabilitation". In: *J Pediatr Gastroenterol Nutr* 51.5 (2010), pp. 638–44. ISSN: 1536-4801 (Electronic) 0277-2116 (Print) 0277-2116 (Linking). doi: [10.1097/MPG.0b013e3181eb3128](https://doi.org/10.1097/MPG.0b013e3181eb3128).
- [118] S. D. Johnston, M. Smye, R. G. Watson, S. A. McMillan, E. R. Trimble, and A. H. Love. "Lactulose-mannitol intestinal permeability test: a useful screening test for adult coeliac disease". In: *Ann Clin Biochem* 37 (Pt 4) (2000), pp. 512–9. ISSN: 0004-5632 (Print) 0004-5632 (Linking). doi: [10.1177/000456320003700413](https://doi.org/10.1177/000456320003700413).
- [119] F. Andre, C. Andre, Y. Emery, J. Forichon, L. Descos, and Y. Minaire. "Assessment of the lactulose-mannitol test in Crohn's disease". In: *Gut* 29.4 (1988), pp. 511–5. ISSN: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.29.4.511](https://doi.org/10.1136/gut.29.4.511).
- [120] J. K. Marshall, M. Thabane, A. X. Garg, W. Clark, J. Meddings, S. M. Collins, and W. E. L. Investigators. "Intestinal permeability in patients with irritable bowel syndrome after a waterborne outbreak of acute gastroenteritis in Walkerton, Ontario". In: *Aliment Pharmacol Ther* 20.11-12 (2004), pp. 1317–22. ISSN: 0269-2813 (Print) 0269-2813 (Linking). doi: [10.1111/j.1365-2036.2004.02284.x](https://doi.org/10.1111/j.1365-2036.2004.02284.x).
- [121] A. Wesselius-de Casparis, S. Braadbaart, G. Bergh-Bohlken, and M. Mimica. "Treatment of chronic constipation with lactulose syrup: results of a double-blind study". In: *gut* 9 (1968), pp. 84–86. doi: <https://doi.org/10.1136/gut.9.1.84>.
- [122] M. Portalatin and N. Winstead. "Medical management of constipation". In: *Clin Colon Rectal Surg* 25.1 (2012), pp. 12–9. ISSN: 1531-0043 (Print) 1530-9681 (Electronic) 1530-9681 (Linking). doi: [10.1055/s-0032-1301754](https://doi.org/10.1055/s-0032-1301754).
- [123] A. Vojdani. "For the assessment of intestinal permeability, size matters". In: *Altern Ther Health Med* 19.1 (2013), pp. 12–24. ISSN: 1078-6791 (Print) 1078-6791 (Linking).
- [124] C. Guerrero, C. Vera, and A. Illanes. "Lactulose". In: *Handbook of Food Bioactive Ingredients* (2023), pp. 1413–1448. doi: https://doi.org/10.1007/978-3-031-28109-9_38.
- [125] M. I. Ordiz, C. Davitt, K. Stephenson, S. Agapova, O. Divala, N. Shaikh, and M. J. Manary. "EB 2017 Article: Interpretation of the lactulose:mannitol test in rural Malawian children at risk for perturbations in intestinal permeability". In: *Exp Biol Med (Maywood)* 243.8 (2018), pp. 677–683. ISSN: 1535-3699 (Electronic) 1535-3702 (Print) 1535-3699 (Linking). doi: [10.1177/1535370218768508](https://doi.org/10.1177/1535370218768508).
- [126] R. M. van Elburg, J. J. Uil, C. J. Mulder, and H. S. Heymans. "Intestinal permeability in patients with coeliac disease and relatives of patients with coeliac disease". In: *Gut* 34.3 (1993), pp. 354–7. ISSN: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.34.3.354](https://doi.org/10.1136/gut.34.3.354).
- [127] P. Munkholm, E. Langholz, D. Hollander, K. Thornberg, M. Orholm, K. D. Katz, and V. Binder. "Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first

- degree relatives". In: *Gut* 35.1 (1994), pp. 68–72. ISSN: 0017-5749 (Print) 1458-3288 (Electronic) 0017-5749 (Linking). doi: [10.1136/gut.35.1.68](https://doi.org/10.1136/gut.35.1.68).
- [128] M. N. Kosek, G. O. Lee, R. L. Guerrant, et al. "Age and Sex Normalization of Intestinal Permeability Measures for the Improved Assessment of Enteropathy in Infancy and Early Childhood: Results From the MAL-ED Study". In: *J Pediatr Gastroenterol Nutr* 65.1 (2017), pp. 31–39. ISSN: 1536-4801 (Electronic) 0277-2116 (Linking). doi: [10.1097/MPG.0000000000001610](https://doi.org/10.1097/MPG.0000000000001610).
- [129] L. D. Juby, J. Rothwell, and A. T. Axon. "Lactulose/mannitol test: an ideal screen for celiac disease". In: *Gastroenterology* 96.1 (1989), pp. 79–85. ISSN: 0016-5085 (Print) 0016-5085 (Linking). doi: [10.1016/0016-5085\(89\)90767-1](https://doi.org/10.1016/0016-5085(89)90767-1).
- [130] M. S. Barboza Junior, T. M. Silva, R. L. Guerrant, and A. A. Lima. "Measurement of intestinal permeability using mannitol and lactulose in children with diarrheal diseases". In: *Braz J Med Biol Res* 32.12 (1999), pp. 1499–504. ISSN: 0100-879X (Print) 0100-879X (Linking). doi: [10.1590/s0100-879x1999001200008](https://doi.org/10.1590/s0100-879x1999001200008).
- [131] M.-E. N. Investigators. "The MAL-ED study: a multinational and multidisciplinary approach to understand the relationship between enteric pathogens, malnutrition, gut physiology, physical growth, cognitive development, and immune responses in infants and children up to 2 years of age in resource-poor environments". In: *Clin Infect Dis* 59 Suppl 4 (2014), S193–206. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). doi: [10.1093/cid/ciu653](https://doi.org/10.1093/cid/ciu653).
- [132] M. R. Khan, W. A. Faubion, R. Dyer, R. Singh, J. J. Larson, and I. Absah. "Role of Lactulose Rhamnose Permeability Test in Assessing Small Bowel Mucosal Damage in Children with Celiac Disease". In: *Glob Pediatr Health* 7 (2020), p. 2333794X20969278. ISSN: 2333-794X (Electronic) 2333-794X (Linking). doi: [10.1177/2333794X20969278](https://doi.org/10.1177/2333794X20969278).
- [133] K. van Wijck, T. J. Verlinden, H. M. van Eijk, J. Dekker, W. A. Buurman, C. H. Dejong, and K. Lenaerts. "Novel multi-sugar assay for site-specific gastrointestinal permeability analysis: a randomized controlled crossover trial". In: *Clin Nutr* 32.2 (2013), pp. 245–51. ISSN: 1532-1983 (Electronic) 0261-5614 (Linking). doi: [10.1016/j.clnu.2012.06.014](https://doi.org/10.1016/j.clnu.2012.06.014).
- [134] M. A. van Nieuwenhoven, B. J. Geerling, N. E. Deutz, F. Brouns, and R. J. Brummer. "The sensitivity of the lactulose/rhamnose gut permeability test". In: *Eur J Clin Invest* 29.2 (1999), pp. 160–5. ISSN: 0014-2972 (Print) 0014-2972 (Linking). doi: [10.1046/j.1365-2362.1999.00421.x](https://doi.org/10.1046/j.1365-2362.1999.00421.x).
- [135] W. A. Faubion, M. Camilleri, J. A. Murray, et al. "Improving the detection of environmental enteric dysfunction: a lactulose, rhamnose assay of intestinal permeability in children aged under 5 years exposed to poor sanitation and hygiene". In: *BMJ Glob Health* 1.1 (2016), e000066. ISSN: 2059-7908 (Print) 2059-7908 (Electronic) 2059-7908 (Linking). doi: [10.1136/bmjgh-2016-000066](https://doi.org/10.1136/bmjgh-2016-000066).
- [136] Z. Jamil, N. T. Iqbal, R. Idress, et al. "Gut integrity and duodenal enteropathogen burden in undernourished children with environmental enteric dysfunction". In: *PLoS Negl Trop Dis* 15.7 (2021), e0009584. ISSN: 1935-2735 (Electronic) 1935-2727 (Print) 1935-2727 (Linking). doi: [10.1371/journal.pntd.0009584](https://doi.org/10.1371/journal.pntd.0009584).
- [137] L. Holtz, J. Hoffmann, L. Linneman, M. He, T. Smyrk, T. C. Liu, N. Shaikh, C. Rodrigues, R. Dyer, and R. Singh. "Rhamnose is superior to mannitol as a monosaccharide in the dual sugar absorption test: a prospective randomized study in children with treatment-naïve celiac disease". In: *Frontiers Pediatric* 10 (2022). doi: <https://doi.org/10.3389/fped.2022.874116>.
- [138] I. Cobden, I. Hamilton, J. Rothwell, and A. T. Axon. "Cellobiose/mannitol test: physiological properties of probe molecules and influence of extraneous factors". In: *Clin Chim Acta* 148.1 (1985), pp. 53–62. ISSN: 0009-8981 (Print) 0009-8981 (Linking). doi: [10.1016/0009-8981\(85\)90300-6](https://doi.org/10.1016/0009-8981(85)90300-6).

- [139] A. Mishra and G. K. Makharia. "Techniques of functional and motility test: how to perform and interpret intestinal permeability". In: *J Neurogastroenterol Motil* 18.4 (2012), pp. 443–7. ISSN: 2093-0887 (Electronic) 2093-0879 (Print) 2093-0879 (Linking). doi: [10.5056/jnm.2012.18.4.443](https://doi.org/10.5056/jnm.2012.18.4.443).
- [140] M. Camilleri, A. Nadeau, J. Lamsam, S. L. Nord, M. Ryks, D. Burton, S. Sweetser, A. R. Zinsmeister, and R. Singh. "Understanding measurements of intestinal permeability in healthy humans with urine lactulose and mannitol excretion". In: *Neurogastroenterol Motil* 22.1 (2010), e15–26. ISSN: 1365-2982 (Electronic) 1350-1925 (Print) 1350-1925 (Linking). doi: [10.1111/j.1365-2982.2009.01361.x](https://doi.org/10.1111/j.1365-2982.2009.01361.x).
- [141] I. R. Sequeira, R. G. Lentle, M. C. Kruger, and R. D. Hurst. "Standardising the lactulose mannitol test of gut permeability to minimise error and promote comparability". In: *PLoS One* 9.6 (2014), e99256. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). doi: [10.1371/journal.pone.0099256](https://doi.org/10.1371/journal.pone.0099256).
- [142] J. Gan, S. Nazarian, J. Teare, A. Darzi, H. Ashrafiyan, and A. Thompson. "A case for improved assessment of gut permeability: a meta-analysis quantifying the lactulose:mannitol ratio in coeliac and Crohn's disease". In: *BMC Gastroenterology* 22.16 (2022). doi: <https://doi.org/10.1186/s12876-021-02082-z>.
- [143] M. Camilleri. "Leaky gut: mechanisms, measurement and clinical implications in humans". In: *Gut* 68.8 (2019), pp. 1516–26. doi: <https://doi.org/10.1136/gutjnl-2019-318427>.
- [144] L. Blomquist, T. Bark, G. Hedenborg, and A. Norman. "Evaluation of the lactulose/manitol and 51Cr-ethylenediaminetetraacetic acid/14C-mannitol methods for intestinal permeability: influence of urinary volume, sex, age, smoking and intestinal disease on marker excretion." In: *Scand Journal of Gastroenterology* 32.8 (1997), pp. 805–12. doi: <https://doi.org/10.3109/00365529708996538>.
- [145] A. M. Lett, A. Lim, C. Skinner, J. Maurice, N. Vergis, A. Darzi, R. Goldin, M. Thursz, and A. Thompson. "Rapid, non-invasive measurement of gastric emptying rate using transcutaneous fluorescence spectroscopy." In: *Biomedical Optics Express* 12 (2021), pp. 4149–64. doi: <https://doi.org/10.1364/BOE.424252>.
- [146] R. Mbuki, S. Chileya, A. Thompson, P. Kelly, and V. Kayamba. "Rapid testing of gut permeability using oral fluorescein and confocal laser endomicroscopy in Zambia adults." In: *Transactions of the Royal Society of Tropical Medicine and Hygiene* 115.10 (2021), pp. 1226–28. doi: <https://doi.org/10.1093/trstmh/trab083>.
- [147] R. Dorshow, C. Hall-Moore, and N. Shaikh. "Measurement of gut permeability using fluorescence tracer agent technology." In: *Science Reports* 7.10888 (2017). doi: <https://doi.org/10.1038/s41598-017-09971-y>.
- [148] R. Dorshow, J. Johnson, M. Debreczeny, R. Riley, J. Shieh, T. Rogers, C. Hall-Moore, N. Shaikh, L. Rouggy-Nickless, and P. Tarr. "Transdermal fluorescence detection of a dual fluorophore system for non-invasive point-of-care gastrointestinal permeability measurement". In: *Biomedical Optics Express* 10 (2019), pp. 5103–16. doi: <https://doi.org/10.1364/BOE.10.005103>.
- [149] Meadibeacon. *Transdermal GFR Monitor - Preclinical MX - MB 2739*. Web Page.
- [150] (ICNIRP), International commission on non-ionizing radiation protection. "ICNIRP guidelines on limits of exposure to laser radiation of wavelengths between 180nm and 1,000 microns." In: *Health Physics* 105.3 (2013), pp. 271–95. doi: <https://doi.org/10.1097/HP.0b013e3182983fd4>.
- [151] M. P. Debreczeny and R. B. Dorshow. "Transdermal optical renal function monitoring in humans: development, verification, and validation of a prototype device". In: *J Biomed Opt* 23.5

- (2018), pp. 1–9. ISSN: 1560-2281 (Electronic) 1083-3668 (Linking). doi: [10.1117/1.JBO.23.5.057003](https://doi.org/10.1117/1.JBO.23.5.057003).
- [152] S. Schulman. *Fluorescence and Phosphorescence Spectroscopy: Physicochemical Principles and Practice*. Elsevier Science and Technology, 1977. ISBN: 9780080204994.
- [153] M. Mycek and B. Pogue. *Handbook of Biomedical Fluorescence*. 2003.
- [154] P. Prasad. *Introduction to Biophotonics*. 2003.
- [155] J. Lakowicz. *Principles of Fluorescence Spectroscopy*. 2006.
- [156] R. Richards-Kortum and E. Sevick-Muraca. “Quantitative optical spectroscopy for tissue diagnosis”. In: *Annu Rev Phys Chem* 47 (1996), pp. 555–606. ISSN: 0066-426X (Print) 0066-426X (Linking). doi: [10.1146/annurev.physchem.47.1.555](https://doi.org/10.1146/annurev.physchem.47.1.555).
- [157] M. Monici. “Cell and tissue autofluorescence research and diagnostic applications”. In: *Biotechnol Annu Rev* 11 (2005), pp. 227–56. ISSN: 1387-2656 (Print) 1387-2656 (Linking). doi: [10.1016/S1387-2656\(05\)11007-2](https://doi.org/10.1016/S1387-2656(05)11007-2).
- [158] M. W. Conklin, P. P. Provenzano, K. W. Eliceiri, R. Sullivan, and P. J. Keely. “Fluorescence lifetime imaging of endogenous fluorophores in histopathology sections reveals differences between normal and tumor epithelium in carcinoma in situ of the breast”. In: *Cell Biochem Biophys* 53.3 (2009), pp. 145–57. ISSN: 1559-0283 (Electronic) 1085-9195 (Print) 1085-9195 (Linking). doi: [10.1007/s12013-009-9046-7](https://doi.org/10.1007/s12013-009-9046-7).
- [159] H. Yang, X. Xiao, X. Zhao, L. Hu, X. Xue, and J. Ye. *Study on fluorescence spectra of Thiamine and Riboflavin*. Conference Paper. 2016. doi: <https://doi.org/10.1051/mateconf/20166303013>.
- [160] V. S. Subramanian, S. B. Subramanya, L. Rapp, J. S. Marchant, T. Y. Ma, and H. M. Said. “Differential expression of human riboflavin transporters -1, -2, and -3 in polarized epithelia: a key role for hRFT-2 in intestinal riboflavin uptake”. In: *Biochim Biophys Acta* 1808.12 (2011), pp. 3016–21. ISSN: 0006-3002 (Print) 0006-3002 (Linking). doi: [10.1016/j.bbamem.2011.08.004](https://doi.org/10.1016/j.bbamem.2011.08.004).
- [161] X. Wu, L. Z. Guo, Y. H. Liu, et al. “Plasma riboflavin fluorescence as a diagnostic marker of mesenteric ischemia-reperfusion injury in rats”. In: *Thromb Res* 223 (2023), pp. 146–154. ISSN: 1879-2472. doi: [10.1016/j.thromres.2023.01.032](https://doi.org/10.1016/j.thromres.2023.01.032).
- [162] A. Resendez, M. Abdul Halim, C. M. Landhage, P. M. Hellstrom, B. Singaram, and D. L. Webb. “Rapid small intestinal permeability assay based on riboflavin and lactulose detected by bis-boronic acid appended benzyl viologens”. In: *Clin Chim Acta* 439 (2015), pp. 115–21. ISSN: 1873-3492 (Electronic) 0009-8981 (Print) 0009-8981 (Linking). doi: [10.1016/j.cca.2014.09.031](https://doi.org/10.1016/j.cca.2014.09.031).
- [163] J. Deal, S. Mayes, C. Browning, S. Hill, P. Rider, C. Boudreaux, T. C. Rich, and S. J. Leavesley. “Identifying molecular contributors to autofluorescence of neoplastic and normal colon sections using excitation-scanning hyperspectral imaging”. In: *J Biomed Opt* 24.2 (2018), pp. 1–11. ISSN: 1560-2281 (Electronic) 1083-3668 (Print) 1083-3668 (Linking). doi: [10.1117/1.JBO.24.2.021207](https://doi.org/10.1117/1.JBO.24.2.021207).
- [164] D. C. De Veld, M. J. Witjes, H. J. Sterenborg, and J. L. Roodenburg. “The status of in vivo autofluorescence spectroscopy and imaging for oral oncology”. In: *Oral Oncol* 41.2 (2005), pp. 117–31. ISSN: 1368-8375 (Print) 1368-8375 (Linking). doi: [10.1016/j.oraloncology.2004.07.007](https://doi.org/10.1016/j.oraloncology.2004.07.007).
- [165] G. Rutili and K. E. Arfors. “Fluorescein-labelled dextran measurement in interstitial fluid in studies of macromolecular permeability”. In: *Microvasc Res* 12.2 (1976), pp. 221–30. ISSN: 0026-2862 (Print) 0026-2862 (Linking). doi: [10.1016/0026-2862\(76\)90019-4](https://doi.org/10.1016/0026-2862(76)90019-4).

- [166] A. Woting and M. Blaut. "Small Intestinal Permeability and Gut-Transit Time Determined with Low and High Molecular Weight Fluorescein Isothiocyanate-Dextran in C3H Mice". In: *Nutrients* 10.6 (2018). issn: 2072-6643. doi: [10.3390/nu10060685](https://doi.org/10.3390/nu10060685).
- [167] L. Wang, C. Llorente, P. Hartmann, A. M. Yang, P. Chen, and B. Schnabl. "Methods to determine intestinal permeability and bacterial translocation during liver disease". In: *J Immunol Methods* 421 (2015), pp. 44–53. issn: 1872-7905 (Electronic) 0022-1759 (Print) 0022-1759 (Linking). doi: [10.1016/j.jim.2014.12.015](https://doi.org/10.1016/j.jim.2014.12.015).
- [168] A. N. de Belder and K. O. Wik. "Preparation and properties of fluorescein-labelled hyaluronate". In: *Carbohydr Res* 44.2 (1975), pp. 251–7. issn: 0008-6215 (Print) 0008-6215 (Linking). doi: [10.1016/s0008-6215\(00\)84168-3](https://doi.org/10.1016/s0008-6215(00)84168-3).
- [169] Y. Olsson, E. Svensjo, K. E. Arfors, and D. Hultstrom. "Fluorescein labelled dextrans as tracers for vascular permeability studies in the nervous system". In: *Acta Neuropathol* 33.1 (1975), pp. 45–50. issn: 0001-6322 (Print) 0001-6322 (Linking). doi: [10.1007/BF00685963](https://doi.org/10.1007/BF00685963).
- [170] T. C. Brelje, M. W. Wessendorf, and R. L. Sorenson. "Multicolor laser scanning confocal immunofluorescence microscopy: practical application and limitations". In: *Methods Cell Biol* 70 (2002), pp. 165–244. issn: 0091-679X (Print) 0091-679X (Linking). doi: [10.1016/s0091-679x\(02\)70006-x](https://doi.org/10.1016/s0091-679x(02)70006-x).
- [171] P. F. McDonagh and S. K. Williams. "The preparation and use of fluorescent-protein conjugates for microvascular research". In: *Microvasc Res* 27.1 (1984), pp. 14–27. issn: 0026-2862 (Print) 0026-2862 (Linking). doi: [10.1016/0026-2862\(84\)90038-4](https://doi.org/10.1016/0026-2862(84)90038-4).
- [172] T. H. The and T. E. Feltkamp. "Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation". In: *Immunology* 18.6 (1970), pp. 865–73. issn: 0019-2805 (Print) 1365-2567 (Electronic) 0019-2805 (Linking).
- [173] U. Schroder, K. E. Arfors, and O. Tangen. "Stability of fluorescein labeled dextrans in vivo and in vitro". In: *Microvasc Res* 11.1 (1976), pp. 57–66. issn: 0026-2862 (Print) 0026-2862 (Linking).
- [174] TdB Labs. *FITC-dextran*. Catalog. 2024.
- [175] J. Hoffmann, M. Schirner, A. Menrad, and M. R. Schneider. "A highly sensitive model for quantification of in vivo tumor angiogenesis induced by alginate-encapsulated tumor cells". In: *Cancer Res* 57.17 (1997), pp. 3847–51. issn: 0008-5472 (Print) 0008-5472 (Linking).
- [176] S. Purandare, K. Offenbartl, B. Westrom, and S. Bengmark. "Increased gut permeability to fluorescein isothiocyanate-dextran after total parenteral nutrition in the rat". In: *Scand J Gastroenterol* 24.6 (1989), pp. 678–82. issn: 0036-5521 (Print) 0036-5521 (Linking). doi: [10.3109/00365528909093108](https://doi.org/10.3109/00365528909093108).
- [177] J. Liu, P. Y. Teng, W. K. Kim, and T. J. Applegate. "Assay considerations for fluorescein isothiocyanate-dextran (FITC-d): an indicator of intestinal permeability in broiler chickens". In: *Poult Sci* 100.7 (2021), p. 101202. issn: 1525-3171 (Electronic) 0032-5791 (Print) 0032-5791 (Linking). doi: [10.1016/j.psj.2021.101202](https://doi.org/10.1016/j.psj.2021.101202).
- [178] A. K. Gaigalas, L. Li, O. Henderson, R. Vogt, J. Barr, G. Marti, J. Weaver, and A. Schwartz. "The Development of Fluorescence Intensity Standards". In: *J Res Natl Inst Stand Technol* 106.2 (2001), pp. 381–9. issn: 1044-677X (Print) 2165-7254 (Electronic) 1044-677X (Linking). doi: [10.6028/jres.106.015](https://doi.org/10.6028/jres.106.015).
- [179] F. Le Guern, V. Mussard, A. Gaucher, M. Rottman, and D. Prim. "Fluorescein Derivatives as Fluorescent Probes for pH Monitoring along Recent Biological Applications". In: *Int J Mol Sci* 21.23 (2020). issn: 1422-0067 (Electronic) 1422-0067 (Linking). doi: [10.3390/ijms21239217](https://doi.org/10.3390/ijms21239217).
- [180] S. Van Keulen, M. Hom, H. White, E. L. Rosenthal, and F. M. Baik. "The Evolution of Fluorescence - Guided Surgery". In: *Mol Imaging Biol* 25.1 (2023), pp. 36–45. issn: 1860-2002 (Electronic) 1536-1632 (Print) 1536-1632 (Linking). doi: [10.1007/s11307-022-01772-8](https://doi.org/10.1007/s11307-022-01772-8).

- [181] M. Eutick. "Sodium Fluorescein - Colourful Past, Bright Future". In: *Journal of Ophthalmic photography* 28.2 (2006).
- [182] (MHRA) Medicines and Healthcare products Regulatory Agency, MHRA. *Assessment report - Summary of product characteristics - fluorescein sodium 100mg/ml, solution for injection, PL 43956-0002*. Report. Medicines and Healthcare products Regulatory Agency, MHRA., 2024.
- [183] Q. T. Nguyen and R. Y. Tsien. "Fluorescence-guided surgery with live molecular navigation—a new cutting edge". In: *Nat Rev Cancer* 13.9 (2013), pp. 653–62. ISSN: 1474-1768 (Electronic) 1474-175X (Print) 1474-175X (Linking). doi: [10.1038/nrc3566](https://doi.org/10.1038/nrc3566).
- [184] M. J. Noble, H. Cheng, and P. M. Jacobs. "Oral fluorescein and cystoid macular oedema: detection in aphakic and pseudophakic eyes". In: *Br J Ophthalmol* 68.4 (1984), pp. 221–4. ISSN: 0007-1161 (Print) 1468-2079 (Electronic) 0007-1161 (Linking). doi: [10.1136/bjo.68.4.221](https://doi.org/10.1136/bjo.68.4.221).
- [185] S. Dithmar and F. Holz. *Fluorescence Angiography in Ophthalmology*. 2008.
- [186] S. Ruia and K. Tripathy. "Fluorescein Angiography". In: *StatPearls*. Treasure Island (FL), 2024.
- [187] S. Weiss and T. Papakostas. "Fluorescein Angiography". In: *Springer - Albert and Jakobiec's Principles and Practice of Ophthalmology*. (2021), pp. 1–24. doi: https://doi.org/10.1007/978-3-319-90495-5_138-2.
- [188] M. J. Amador-Patarroyo, T. Lin, A. Meshi, K. C. Dans, K. Chen, S. Borooah, N. Molano-Gonzalez, J. A. Diaz-Rojas, and W. R. Freeman. "Identifying the factors for improving quality of oral fluorescein angiography". In: *Br J Ophthalmol* 104.4 (2020), pp. 504–508. ISSN: 1468-2079 (Electronic) 0007-1161 (Linking). doi: [10.1136/bjophthalmol-2019-314187](https://doi.org/10.1136/bjophthalmol-2019-314187).
- [189] K. Kuroda, H. Kinouchi, K. Kanemaru, Y. Nishiyama, M. Ogiwara, H. Yoshioka, and T. Horikoshi. "Intra-arterial injection fluorescein videoangiography in aneurysm surgery". In: *Neurosurgery* 72.2 Suppl Operative (2013), pp. 141–50, discussion 150. ISSN: 1524-4040 (Electronic) 0148-396X (Linking). doi: [10.1227/NEU.0b013e3182752f32](https://doi.org/10.1227/NEU.0b013e3182752f32).
- [190] M. Wallace, A. Meining, M. Canto, et al. "The safety of intravenous fluorescein for confocal laser endomicroscopy in the gastrointestinal tract." In: *Alimentary Pharmacology and Therapeutics* 31 (2010), pp. 548–52. doi: <https://doi.org/10.1111/j.1365-2036.2009.04207.x>.
- [191] T. Cwalinski, W. Polom, L. Marano, G. Roviello, A. D'Angelo, N. Cwalina, M. Matuszewski, F. Roviello, J. Jaskiewicz, and K. Polom. "Methylene Blue-Current Knowledge, Fluorescent Properties, and Its Future Use". In: *J Clin Med* 9.11 (2020). ISSN: 2077-0383 (Print) 2077-0383 (Electronic) 2077-0383 (Linking). doi: [10.3390/jcm9113538](https://doi.org/10.3390/jcm9113538).
- [192] J. Huang, X. Gao, M. Wang, et al. "Prophylactic Administration with Methylene Blue Improves Hemodynamic Stabilization During Obstructive Jaundice-Related Diseases' Operation: a Blinded Randomized Controlled Trial". In: *J Gastrointest Surg* 27.9 (2023), pp. 1837–1845. ISSN: 1873-4626 (Electronic) 1091-255X (Print) 1091-255X (Linking). doi: [10.1007/s11605-022-05499-3](https://doi.org/10.1007/s11605-022-05499-3).
- [193] M. Ibarra-Estrada, E. Kattan, P. Aguilera-Gonzalez, et al. "Early adjunctive methylene blue in patients with septic shock: a randomized controlled trial". In: *Crit Care* 27.1 (2023), p. 110. ISSN: 1466-609X (Electronic) 1364-8535 (Print) 1364-8535 (Linking). doi: [10.1186/s13054-023-04397-7](https://doi.org/10.1186/s13054-023-04397-7).
- [194] E. Bistas and D. K. Sanghavi. "Methylene Blue". In: *StatPearls*. Treasure Island (FL), 2024.
- [195] V. Cagno, C. Medaglia, A. Cerny, T. Cerny, A. C. Zwygart, E. Cerny, and C. Tapparel. "Methylene Blue has a potent antiviral activity against SARS-CoV-2 and H1N1 influenza virus in the absence of UV-activation in vitro". In: *Sci Rep* 11.1 (2021), p. 14295. ISSN: 2045-2322 (Electronic) 2045-2322 (Linking). doi: [10.1038/s41598-021-92481-9](https://doi.org/10.1038/s41598-021-92481-9).

- [196] F. B. Borghi-Pangoni, M. V. Junqueira, S. B. de Souza Ferreira, L. L. Silva, B. R. Rabello, W. Caetano, A. Diniz, and M. L. Bruschi. "Screening and In Vitro Evaluation of Mucoadhesive Thermoresponsive System Containing Methylene Blue for Local Photodynamic Therapy of Colorectal Cancer". In: *Pharm Res* 33.3 (2016), pp. 776–91. ISSN: 1573-904X (Electronic) 0724-8741 (Linking). doi: [10.1007/s11095-015-1826-8](https://doi.org/10.1007/s11095-015-1826-8).
- [197] U. Miglani, P. Laul, H. Rajpal, V. K. Kadam, and G. Chowdhary. "Methylene Blue Use: Is It Safe?" In: *J Obstet Gynaecol India* 69.1 (2019), pp. 97–98. ISSN: 0971-9202 (Print) 0975-6434 (Electronic) 0975-6434 (Linking). doi: [10.1007/s13224-018-1168-7](https://doi.org/10.1007/s13224-018-1168-7).
- [198] P. R. Ginimuge and S. D. Jyothi. "Methylene blue: revisited". In: *J Anaesthesiol Clin Pharmacol* 26.4 (2010), pp. 517–20. ISSN: 2231-2730 (Electronic) 0970-9185 (Print) 0970-9185 (Linking).
- [199] Z. Wang, L. Mei, X. Yang, T. Jiang, T. Sun, Y. Su, Y. Wu, and Y. Ji. "Near-infrared fluorophores methylene blue for targeted imaging of the stomach in intraoperative navigation". In: *Front Bioeng Biotechnol* 11 (2023), p. 1172073. ISSN: 2296-4185 (Print) 2296-4185 (Electronic) 2296-4185 (Linking). doi: [10.3389/fbioe.2023.1172073](https://doi.org/10.3389/fbioe.2023.1172073).
- [200] J. Palma, W. Kim, M. Serafino, S. Kim, J. Jo, P. Charoenphol, and B. Applegate. *PLGA Encapsulated Methylene Blue as a Contrast Agent for Optical Coherence Tomography*. Conference Paper. 2019. doi: <https://doi.org/10.1109/IPCon.2019.8908365>.
- [201] C. Zhang, D. Jiang, B. Huang, C. Wang, L. Zhao, X. Xie, Z. Zhang, K. Wang, J. Tian, and Y. Luo. "Methylene Blue-Based Near-Infrared Fluorescence Imaging for Breast Cancer Visualization in Resected Human Tissues". In: *Technol Cancer Res Treat* 18 (2019), p. 1533033819894331. ISSN: 1533-0338. doi: [10.1177/1533033819894331](https://doi.org/10.1177/1533033819894331).
- [202] S. Boonhai, K. Bootdee, W. Saisorn, K. Takkavatakarn, P. Sitticharoenchai, S. Tungsanga, K. Tiranathanagul, and A. Leelahavanichkul. "TMAO reductase, a biomarker for gut permeability defect induced inflammation, in mouse model of chronic kidney disease and dextran sulfate solution-induced mucositis". In: *Asian Pac J Allergy Immunol* 41.2 (2023), pp. 168–178. ISSN: 0125-877X (Print) 0125-877X (Linking). doi: [10.12932/AP-100321-1084](https://doi.org/10.12932/AP-100321-1084).
- [203] P. Nejdfors, J. Konyves, T. Davidsson, M. Ekelund, W. Mansson, and B. R. Westrom. "Permeability of intestinal mucosa from urinary reservoirs in man and rat". In: *BJU Int* 86.9 (2000), pp. 1058–63. ISSN: 1464-4096 (Print) 1464-4096 (Linking). doi: [10.1046/j.1464-410x.2000.00945.x](https://doi.org/10.1046/j.1464-410x.2000.00945.x).
- [204] M. K. Hamilton, G. Boudry, D. G. Lemay, and H. E. Raybould. "Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent". In: *Am J Physiol Gastrointest Liver Physiol* 308.10 (2015), G840–51. ISSN: 1522-1547 (Electronic) 0193-1857 (Print) 0193-1857 (Linking). doi: [10.1152/ajpgi.00029.2015](https://doi.org/10.1152/ajpgi.00029.2015).
- [205] A. F. Duff, L. R. Bielke, and A. E. Relling. "Technical note: fluorescein as an indicator of enteric mucosal barrier function in preruminant lambs". In: *J Anim Sci* 98.7 (2020). ISSN: 1525-3163 (Electronic) 0021-8812 (Print) 0021-8812 (Linking). doi: [10.1093/jas/skaa198](https://doi.org/10.1093/jas/skaa198).
- [206] M. H. Chang and W. Karasov. "Absorption and paracellular visualization of fluorescein, a hydrosoluble probe, in intact house sparrows (*Passer domesticus*)". In: *Zoology* 107 (2004), pp. 121–33. doi: <https://doi.org/10.1016/j.zool.2004.03.001>.
- [207] M. W. Shahid, J. E. Crook, A. Meining, A. Perchant, A. Buchner, V. Gomez, and M. B. Wallace. "Exploring the optimal fluorescein dose in probe-based confocal laser endomicroscopy for colonic imaging". In: *J Interv Gastroenterol* 1.4 (2011), pp. 166–171. ISSN: 2154-1280 (Print) 2154-1299 (Electronic) 2154-1280 (Linking). doi: [10.4161/jig.19953](https://doi.org/10.4161/jig.19953).
- [208] Meadibeacon. *The Transdermal GFR Measurement System (TGFR)*. Web Page. 2024.
- [209] J. Gan, E. Monfort-Sanchez, J. Avery, O. Barouti, J. Hoare, H. Ashrafian, A. Darzi, and A. Thompson. "Non-invasive assessment of intestinal permeability in healthy volunteers using

- transcutaneous fluorescence spectroscopy". In: *Method and Applications in Fluorescence* 10.4 (2022). doi: <https://doi.org/10.1088/2050-6120/ac9513>.
- [210] S. Bischoff, G. Barbara, W. Buurman, T. Ockhuizen, J. Schulzke, M. Serino, H. Tilg, A. Watson, and J. Wells. "Intestinal permeability - a new target for disease prevention and therapy." In: *BMC Gastroenterology* 14.189 (2014). doi: <https://doi.org/10.1186/s12876-014-0189-7>.
- [211] Y. Hu, Y. Wang, F. Tan, and W. Yang. "Regulation of paracellular permeability: factors and mechanisms." In: *Molecular Biology Reports* 40 (2013), pp. 6123–42. doi: <https://doi.org/10.1007/s11033-013-2724-y>.
- [212] E. A. Mayer, K. Tillisch, and A. Gupta. "Gut: brain axis and the microbiota". In: *The journal of Clinical Investigation* 125.3 (2015), pp. 926–38. doi: <https://doi.org/10.1172/JCI76304>.
- [213] Q. Fu, H. Wang, M. Xia, B. Deng, H. Shen, G. Ji, G. Li, and Y. Xie. "The effect of phytic acid on tight junctions in the human intestinal Caco-2 cell line and its mechanism". In: *European Journal of Pharmaceutical Sciences* 80 (2015), pp. 1–8. doi: <https://doi.org/10.1016/j.ejps.2015.09.009>.
- [214] S. Maher, L. Feighery, and D. Brayden. "Melittin as an Epithelial Permeability Enhancer I: Investigation of its mechanism of action in Caco-2 monolayers." In: *Pharmaceutical Research* 24 (2007), pp. 1336–45. doi: <https://doi.org/10.1007/s11095-007-9288-2>.
- [215] J. Crapser, R. Ritzel, R. Verma, et al. "Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice". In: *Aging* 8 (2016), pp. 1049–60. doi: <https://doi.org/10.18632/aging.100952>.
- [216] M. Laker and I. Menzies. "Increase in human intestinal permeability following ingestion of hypertonic solutions." In: *The journal of Physiology* 265 (1977). doi: <https://doi.org/10.1113/jphysiol.1977.sp011750>.
- [217] P. Wheeler, I. Menzies, and B. Creamer. "Effect of hyperosmolar stimuli and coeliac disease on the permeability of the human gastrointestinal tract". In: *Clinical Science and Molecular Medicine* 54.5 (1978), pp. 495–501. doi: <https://doi.org/10.1042/cs0540495>.
- [218] (ICNIRP), International commission on non-ionizing radiation protection. "Guidelines on limits of exposure to laser radiation of wavelengths between 180 nm and 1,000 microns". In: *Health Physics* 71.5 (1996), pp. 804–819.
- [219] (ICNIRP), International commission on non-ionizing radiation protection. "Revision of guidelines on limits of exposure to laser radiation of wavelengths between 400nm and 1.4 microns". In: *Health Physics* 79.4 (2000), pp. 431–40.
- [220] A. Thompson. *Non-invasive transcutaneous spectroscopy for the assessment of gut permeability*. Report. 2018. doi: https://classic.clinicaltrials.gov/ProvidedDocs/39/NCT03434639/Prot_SAP_008.pdf.
- [221] T. Ahmed. "Pharmacokinetics of drugs following IV bolus, IV infusion and Oral administration". In: *InTech* (2015). doi: [10.5772/61573](https://doi.org/10.5772/61573).
- [222] D. Grotte, V. Mattox, and R. Brubaker. "Fluorescent, physiological and pharmacokinetic properties of fluorescein glucuronide". In: *Exp Eye Res* 40.1 (1985), pp. 23–33. ISSN: 0014-4835 (Print) 0014-4835 (Linking). doi: [10.1016/0014-4835\(85\)90105-8](https://doi.org/10.1016/0014-4835(85)90105-8).
- [223] A. Thompson, T. Herling, M. Kubankova, A. Vysniauskas, T. Knowles, and M. K. Kuimova. "Molecular rotors provide insights into microscopic structural changes during protein aggregation". In: *Journal of Physical chemistry* 119.32 (2015), pp. 10170–10179. doi: <https://doi.org/10.1021/acs.jpcb.5b05099>.
- [224] M. Digman, V. Caiolfa, M. Zamai, and E. Gratton. "The phasor approach to fluorescence lifetime imaging analysis". In: *Biophysical Journal* 94.2 (2008), pp. L14–L16. doi: <https://doi.org/10.1529/biophysj.107.120154>.

- [225] A. Mariette and R. Khanna. "Support vector machines for classification". In: *Efficient learning machines* (2015), pp. 39–66. doi: https://doi.org/10.1007/978-1-4302-5990-9_3.
- [226] R. Kousovista, G. Karali, and V. Karalis. "Modeling the double peak phenomenon in drug absorption kinetics: the case of amisulpride". In: *BioMedInformatics* 3.1 (2023), pp. 177–192. doi: <https://doi.org/10.3390/biomedinformatics3010013>.
- [227] A. Madison and J. Kiecolt-Glaser. "Stress, depression, diet, and the gut microbiota: human-bacteria interactions at the core of psychoneuroimmunology and nutrition". In: *Current opinion in behavioral sciences* 28 (2019), pp. 105–110. doi: <https://doi.org/10.1016/j.cobeha.2019.01.011>.
- [228] B. Keirns, N. Koemel, C. Sciarrillo, K. Anderson, and S. Emerson. "Exercise and intestinal permeability: another form of exercise-induced hormesis?" In: *Gastrointestinal and liver physiology* (2020), pp. 105–110. doi: <https://doi.org/10.1152/ajpgi.00232.2020>.
- [229] E. Monfort-Sanchez, J. Avery, J. Gan, et al. "A compact fluorescence sensor for low-cost non-invasive monitoring of gut permeability in undernutrition". In: *SPIE Proceedings* (2023). doi: <https://doi.org/10.1117/12.2649030>.
- [230] E. M. Sanchez, J. Avery, J. Gan, et al. "Transcutaneous fluorescence spectroscopy: development and characterization of a compact, portable, and fiber-optic sensor". In: *J Biomed Opt* 29.2 (2024), p. 027003. ISSN: 1560-2281 (Electronic) 1083-3668 (Print) 1083-3668 (Linking). doi: [10.1117/1.JBO.29.2.027003](https://doi.org/10.1117/1.JBO.29.2.027003).
- [231] D. M. Denno, K. VanBuskirk, Z. C. Nelson, C. A. Musser, D. C. Hay Burgess, and P. I. Tarr. "Use of the lactulose to mannitol ratio to evaluate childhood environmental enteric dysfunction: a systematic review". In: *Clin Infect Dis* 59 Suppl 4 (2014), S213–9. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). doi: [10.1093/cid/ciu541](https://doi.org/10.1093/cid/ciu541).
- [232] M. Runciman, J. Avery, M. Zhao, A. Darzi, and G. P. Mylonas. "Deployable, Variable Stiffness, Cable Driven Robot for Minimally Invasive Surgery". In: *Front Robot AI* 6 (2019), p. 141. ISSN: 2296-9144 (Electronic) 2296-9144 (Linking). doi: [10.3389/frobt.2019.00141](https://doi.org/10.3389/frobt.2019.00141).
- [233] M. Goldman. *Universal Classification of Skin Type - Simplified Facial Rejuvenation*. 2008. doi: https://doi.org/10.1007/978-3-540-71097-4_3.
- [234] A. G. Pothen and M. Parmar. "Fluorescein". In: *StatPearls*. Treasure Island (FL), 2024.
- [235] R. Farre, M. Fiorani, S. Abdu Rahiman, and G. Matteoli. "Intestinal Permeability, Inflammation and the Role of Nutrients". In: *Nutrients* 12.4 (2020). ISSN: 2072-6643 (Electronic) 2072-6643 (Linking). doi: [10.3390/nu12041185](https://doi.org/10.3390/nu12041185).
- [236] K. Khoshbin and M. Camilleri. "Effects of dietary components on intestinal permeability in health and disease". In: *Am J Physiol Gastrointest Liver Physiol* 319.5 (2020), G589–G608. ISSN: 1522-1547 (Electronic) 0193-1857 (Print) 0193-1857 (Linking). doi: [10.1152/ajpgi.00245.2020](https://doi.org/10.1152/ajpgi.00245.2020).
- [237] A. S. Rao, M. Camilleri, D. J. Eckert, I. Busciglio, D. D. Burton, M. Ryks, B. S. Wong, J. Lam-sam, R. Singh, and A. R. Zinsmeister. "Urine sugars for in vivo gut permeability: validation and comparisons in irritable bowel syndrome-diarrhea and controls". In: *Am J Physiol Gastrointest Liver Physiol* 301.5 (2011), G919–28. ISSN: 1522-1547 (Electronic) 0193-1857 (Print) 0193-1857 (Linking). doi: [10.1152/ajpgi.00168.2011](https://doi.org/10.1152/ajpgi.00168.2011).
- [238] M. Kosek, R. L. Guerrant, G. Kang, et al. "Assessment of environmental enteropathy in the MAL-ED cohort study: theoretical and analytic framework". In: *Clin Infect Dis* 59 Suppl 4. Suppl 4 (2014), S239–47. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). doi: [10.1093/cid/ciu457](https://doi.org/10.1093/cid/ciu457).
- [239] J. R. Lightdale, Q. Y. Liu, B. Sahn, D. M. Troendle, M. Thomson, D. S. Fishman, N. Endoscopy, and C. Procedures. "Pediatric Endoscopy and High-risk Patients: A Clinical Report From the NASPGHAN Endoscopy Committee". In: *J Pediatr Gastroenterol Nutr* 68.4 (2019), pp. 595–

606. ISSN: 1536-4801 (Electronic) 0277-2116 (Print) 0277-2116 (Linking). doi: [10.1097/MPG.0000000000002277](https://doi.org/10.1097/MPG.0000000000002277).
- [240] L. Norvang, T. Milner, J. Nelson, M. Berns, and L. Svaasand. "Skin pigmentation characterized by visible reflectance measurements". In: *Lasers in Medical Science* 12.2 (1997), pp. 99–112. doi: <https://doi.org/10.1007/bf02763978>.
- [241] S. Serra and P. A. Jani. "An approach to duodenal biopsies". In: *J Clin Pathol* 59.11 (2006), pp. 1133–50. ISSN: 0021-9746 (Print) 1472-4146 (Electronic) 0021-9746 (Linking). doi: [10.1136/jcp.2005.031260](https://doi.org/10.1136/jcp.2005.031260).
- [242] O. Kwon, T. S. Han, and M. Y. Son. "Intestinal Morphogenesis in Development, Regeneration, and Disease: The Potential Utility of Intestinal Organoids for Studying Compartmentalization of the Crypt-Villus Structure". In: *Front Cell Dev Biol* 8 (2020), p. 593969. ISSN: 2296-634X (Print) 2296-634X (Electronic) 2296-634X (Linking). doi: [10.3389/fcell.2020.593969](https://doi.org/10.3389/fcell.2020.593969).
- [243] J. Khan and M. Islam. "Morphology of the intestinal barrier in different physiological and pathological conditions". In: *InTech* (2012). doi: [10.5772/50659](https://doi.org/10.5772/50659).
- [244] B. Amadi, K. Zyambo, K. Chandwe, et al. "Adaptation of the small intestine to microbial enteropathogens in Zambian children with stunting". In: *Nat Microbiol* 6.4 (2021), pp. 445–454. ISSN: 2058-5276 (Electronic) 2058-5276 (Linking). doi: [10.1038/s41564-020-00849-w](https://doi.org/10.1038/s41564-020-00849-w).
- [245] P. J. Wahab, J. W. Meijer, and C. J. Mulder. "Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery". In: *Am J Clin Pathol* 118.3 (2002), pp. 459–63. ISSN: 0002-9173 (Print) 0002-9173 (Linking). doi: [10.1309/EVXT-851X-WHLC-RLX9](https://doi.org/10.1309/EVXT-851X-WHLC-RLX9).
- [246] D. C. Rubin and M. S. Levin. "Mechanisms of intestinal adaptation". In: *Best Pract Res Clin Gastroenterol* 30.2 (2016), pp. 237–48. ISSN: 1532-1916 (Electronic) 1521-6918 (Print) 1521-6918 (Linking). doi: [10.1016/j.bpg.2016.03.007](https://doi.org/10.1016/j.bpg.2016.03.007).
- [247] D. Shaw, K. Gohil, and M. D. Basson. "Intestinal mucosal atrophy and adaptation". In: *World J Gastroenterol* 18.44 (2012), pp. 6357–75. ISSN: 2219-2840 (Electronic) 1007-9327 (Print) 1007-9327 (Linking). doi: [10.3748/wjg.v18.i44.6357](https://doi.org/10.3748/wjg.v18.i44.6357).
- [248] M. A. Miller, H. P. Parkman, J. L. Urbain, K. L. Brown, D. J. Donahue, L. C. Knight, A. H. Maurer, and R. S. Fisher. "Comparison of scintigraphy and lactulose breath hydrogen test for assessment of orocecal transit: lactulose accelerates small bowel transit". In: *Dig Dis Sci* 42.1 (1997), pp. 10–8. ISSN: 0163-2116 (Print) 0163-2116 (Linking). doi: [10.1023/a:1018864400566](https://doi.org/10.1023/a:1018864400566).
- [249] E. Fritz, H. F. Hammer, R. W. Lipp, C. Hogenauer, R. Stauber, and J. Hammer. "Effects of lactulose and polyethylene glycol on colonic transit". In: *Aliment Pharmacol Ther* 21.3 (2005), pp. 259–68. ISSN: 0269-2813 (Print) 0269-2813 (Linking). doi: [10.1111/j.1365-2036.2005.02244.x](https://doi.org/10.1111/j.1365-2036.2005.02244.x).
- [250] L. Schiler. "The therapy of constipation". In: *Alimentary Pharmacology and Therapeutics* 15.6 (2002), pp. 749–763. doi: <https://doi.org/10.1046/j.1365-2036.2001.00982.x>.
- [251] A. Althnian, D. AlSaeed, H. Al-Baity, A. Samha, A. Bin Dris, N. Alzakari, A. Elwafa, and H. Kurdi. "Impact of Dataset Size on Classification performance: an empirical evaluation in the medical domain." In: *Applied Science* 11.2 (2021), p. 796. doi: <https://doi.org/10.3390/app11020796>.
- [252] P. Kelly, E. Besa, K. Zyambo, J. Louis-Auguste, J. Lees, T. Banda, R. Soko, R. Banda, B. Amadi, and A. Watson. "Correction: Endomicroscopic and Transcriptomic Analysis of Impaired Barrier Function and Malabsorption in Environmental Enteropathy". In: *PLoS Negl Trop Dis* 16.6 (2022), e0010505. ISSN: 1935-2735 (Electronic) 1935-2727 (Print) 1935-2727 (Linking). doi: [10.1371/journal.pntd.0010505](https://doi.org/10.1371/journal.pntd.0010505).

- [253] P. S. Chahal, M. J. Neal, and E. M. Kohner. "Metabolism of fluorescein after intravenous administration". In: *Invest Ophthalmol Vis Sci* 26.5 (1985), pp. 764–8. ISSN: 0146-0404 (Print) 0146-0404 (Linking).
- [254] E. A. McKenney, A. R. Hale, J. Anderson, R. Larsen, C. Grant, and R. R. Dunn. "Hidden diversity: comparative functional morphology of humans and other species". In: *PeerJ* 11 (2023), e15148. ISSN: 2167-8359 (Electronic) 2167-8359 (Linking). doi: [10.7717/peerj.15148](https://doi.org/10.7717/peerj.15148).
- [255] H. F. Helander and L. Fandriks. "Surface area of the digestive tract - revisited". In: *Scand J Gastroenterol* 49.6 (2014), pp. 681–9. ISSN: 1502-7708 (Electronic) 0036-5521 (Linking). doi: [10.3109/00365521.2014.898326](https://doi.org/10.3109/00365521.2014.898326).
- [256] P. M. Ueno, R. B. Oria, E. A. Maier, et al. "Alanyl-glutamine promotes intestinal epithelial cell homeostasis in vitro and in a murine model of weanling undernutrition". In: *Am J Physiol Gastrointest Liver Physiol* 301.4 (2011), G612–22. ISSN: 1522-1547 (Electronic) 0193-1857 (Print) 0193-1857 (Linking). doi: [10.1152/ajpgi.00531.2010](https://doi.org/10.1152/ajpgi.00531.2010).
- [257] M. J. Cho, D. P. Thompson, C. T. Cramer, T. J. Vidmar, and J. F. Scieszka. "The Madin Darby canine kidney (MDCK) epithelial cell monolayer as a model cellular transport barrier". In: *Pharm Res* 6.1 (1989), pp. 71–7. ISSN: 0724-8741 (Print) 0724-8741 (Linking). doi: [10.1023/a:1015807904558](https://doi.org/10.1023/a:1015807904558).
- [258] J. Leighton, L. W. Estes, S. Mansukhani, and Z. Brada. "A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium". In: *Cancer* 26.5 (1970), pp. 1022–8. ISSN: 0008-543X (Print) 0008-543X (Linking). doi: [10.1002/1097-0142\(197011\)26:5<1022::aid-cnrc2820260509>3.0.co;2-m](https://doi.org/10.1002/1097-0142(197011)26:5<1022::aid-cnrc2820260509>3.0.co;2-m).
- [259] H. S. U, P. Boerner, M. J. Rindler, L. Chuman, and J. Saier M. H. "Characterization of chemically and virally transformed variants of Madin-Darby canine kidney (MDCK) epithelial cells". In: *J Cell Physiol* 122.2 (1985), pp. 299–307. ISSN: 0021-9541 (Print) 0021-9541 (Linking). doi: [10.1002/jcp.1041220220](https://doi.org/10.1002/jcp.1041220220).
- [260] I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. "Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability". In: *Gastroenterology* 96.3 (1989), pp. 736–49. ISSN: 0016-5085 (Print) 0016-5085 (Linking).
- [261] A. R. Hilgers, R. A. Conradi, and P. S. Burton. "Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa". In: *Pharm Res* 7.9 (1990), pp. 902–10. ISSN: 0724-8741 (Print) 0724-8741 (Linking). doi: [10.1023/a:1015937605100](https://doi.org/10.1023/a:1015937605100).
- [262] J. Balcarova-Stander, S. E. Pfeiffer, S. D. Fuller, and K. Simons. "Development of cell surface polarity in the epithelial Madin-Darby canine kidney (MDCK) cell line". In: *EMBO J* 3.11 (1984), pp. 2687–94. ISSN: 0261-4189 (Print) 1460-2075 (Electronic) 0261-4189 (Linking). doi: [10.1002/j.1460-2075.1984.tb02194.x](https://doi.org/10.1002/j.1460-2075.1984.tb02194.x).
- [263] J. Keemink, O. J. Hedge, V. Bianco, M. Hubert, and C. A. S. Bergstrom. "Comparison of Cellular Monolayers and an Artificial Membrane as Absorptive Membranes in the in vitro Lipolysis-permeation Assay". In: *J Pharm Sci* 111.1 (2022), pp. 175–184. ISSN: 1520-6017 (Electronic) 0022-3549 (Linking). doi: [10.1016/j.xphs.2021.09.009](https://doi.org/10.1016/j.xphs.2021.09.009).
- [264] D. Oltra-Noguera, V. Mangas-Sanjuan, A. Centelles-Sanguesa, I. Gonzalez-Garcia, G. Sanchez-Castano, M. Gonzalez-Alvarez, V. G. Casabo, V. Merino, I. Gonzalez-Alvarez, and M. Bermejo. "Variability of permeability estimation from different protocols of subculture and transport experiments in cell monolayers". In: *J Pharmacol Toxicol Methods* 71 (2015), pp. 21–32. ISSN: 1873-488X (Electronic) 1056-8719 (Linking). doi: [10.1016/j.vascn.2014.11.004](https://doi.org/10.1016/j.vascn.2014.11.004).
- [265] D. A. Volpe. "Drug-permeability and transporter assays in Caco-2 and MDCK cell lines". In: *Future Med Chem* 3.16 (2011), pp. 2063–77. ISSN: 1756-8927 (Electronic) 1756-8919 (Linking). doi: [10.4155/fmc.11.149](https://doi.org/10.4155/fmc.11.149).

- [266] X. Jin, T. L. Luong, N. Reese, et al. "Comparison of MDCK-MDR1 and Caco-2 cell based permeability assays for anti-malarial drug screening and drug investigations". In: *J Pharmacol Toxicol Methods* 70.2 (2014), pp. 188–94. ISSN: 1873-488X (Electronic) 1056-8719 (Linking). doi: [10.1016/j.vascn.2014.08.002](https://doi.org/10.1016/j.vascn.2014.08.002).
- [267] H. Sun, E. C. Chow, S. Liu, Y. Du, and K. S. Pang. "The Caco-2 cell monolayer: usefulness and limitations". In: *Expert Opin Drug Metab Toxicol* 4.4 (2008), pp. 395–411. ISSN: 1742-5255 (Print) 1742-5255 (Linking). doi: [10.1517/17425255.4.4.395](https://doi.org/10.1517/17425255.4.4.395).
- [268] E. Elamin, D. Jonkers, K. Juuti-Uusitalo, S. van Ijzendoorn, F. Troost, H. Duimel, J. Broers, F. Verheyen, J. Dekker, and A. Masclee. "Effects of ethanol and acetaldehyde on tight junction integrity: in vitro study in a three dimensional intestinal epithelial cell culture model". In: *PLoS One* 7.4 (2012), e35008. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). doi: [10.1371/journal.pone.0035008](https://doi.org/10.1371/journal.pone.0035008).
- [269] T. Y. Ma, D. Nguyen, V. Bui, H. Nguyen, and N. Hoa. "Ethanol modulation of intestinal epithelial tight junction barrier". In: *Am J Physiol* 276.4 (1999), G965–74. ISSN: 0002-9513 (Print) 0002-9513 (Linking). doi: [10.1152/ajpgi.1999.276.4.G965](https://doi.org/10.1152/ajpgi.1999.276.4.G965).
- [270] E. Elamin, A. Masclee, F. Troost, H. J. Pieters, D. Keszthelyi, K. Aleksa, J. Dekker, and D. Jonkers. "Ethanol impairs intestinal barrier function in humans through mitogen activated protein kinase signaling: a combined in vivo and in vitro approach". In: *PLoS One* 9.9 (2014), e107421. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). doi: [10.1371/journal.pone.0107421](https://doi.org/10.1371/journal.pone.0107421).
- [271] S. Tria, L. H. Jimison, A. Hama, M. Bongo, and R. M. Owens. "Sensing of EGTA Mediated Barrier Tissue Disruption with an Organic Transistor". In: *Biosensors (Basel)* 3.1 (2013), pp. 44–57. ISSN: 2079-6374 (Print) 2079-6374 (Electronic) 2079-6374 (Linking). doi: [10.3390/bios3010044](https://doi.org/10.3390/bios3010044).
- [272] X. Boulenc, E. Marti, H. Joyeux, C. Roques, Y. Berger, and G. Fabre. "Importance of the paracellular pathway for the transport of a new bisphosphonate using the human CACO-2 monolayers model". In: *Biochem Pharmacol* 46.9 (1993), pp. 1591–600. ISSN: 0006-2952 (Print) 0006-2952 (Linking). doi: [10.1016/0006-2952\(93\)90328-t](https://doi.org/10.1016/0006-2952(93)90328-t).
- [273] P. Artursson. "Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells". In: *J Pharm Sci* 79.6 (1990), pp. 476–82. ISSN: 0022-3549 (Print) 0022-3549 (Linking). doi: [10.1002/jps.2600790604](https://doi.org/10.1002/jps.2600790604).
- [274] H. Bootz-Maoz, A. Simon, S. Del Mare-Roumani, Y. Bennet, E. Toister, H. Romano, D. Zheng, S. Amidror, E. Elinav, and N. Yissachar. "Ex vivo intestinal permeability assay (X-IPA) for tracking barrier function dynamics". In: *NPJ Biofilms Microbiomes* 9.1 (2023), p. 44. ISSN: 2055-5008 (Electronic) 2055-5008 (Linking). doi: [10.1038/s41522-023-00409-0](https://doi.org/10.1038/s41522-023-00409-0).
- [275] National Center for Biotechnology Information. *PubChem Compound Summary for CID 16850, Fluorescein*. Online Database. 2024.
- [276] National library of Medicine. *PubChem Compound Summary for CID 6099, Methylene Blue*. Online Database. 2024. doi: <https://pubchem.ncbi.nlm.nih.gov/compound/Methylene-Blue>.
- [277] A. Seth, S. Basuroy, P. Sheth, and R. K. Rao. "L-Glutamine ameliorates acetaldehyde-induced increase in paracellular permeability in Caco-2 cell monolayer". In: *Am J Physiol Gastrointest Liver Physiol* 287.3 (2004), G510–7. ISSN: 0193-1857 (Print) 0193-1857 (Linking). doi: [10.1152/ajpgi.00058.2004](https://doi.org/10.1152/ajpgi.00058.2004).
- [278] A. Banan, S. Choudhary, Y. Zhang, J. Z. Fields, and A. Keshavarzian. "Ethanol-induced barrier dysfunction and its prevention by growth factors in human intestinal monolayers: evidence for oxidative and cytoskeletal mechanisms". In: *J Pharmacol Exp Ther* 291.3 (1999), pp. 1075–85. ISSN: 0022-3565 (Print) 0022-3565 (Linking).

- [279] B. J. Glasgow. "Fluorescence lifetime imaging microscopy reveals quenching of fluorescein within corneal epithelium". In: *Exp Eye Res* 147 (2016), pp. 12–19. ISSN: 1096-0007 (Electronic) 0014-4835 (Print) 0014-4835 (Linking). doi: [10.1016/j.exer.2016.04.008](https://doi.org/10.1016/j.exer.2016.04.008).
- [280] A. K. Gaigalas, L. Li, O. Henderson, R. Vogt, J. Barr, G. Marti, J. Weaver, and A. Schwartz. "The Development of Fluorescence Intensity Standards". In: *J Res Natl Inst Stand Technol* 106.2 (2001), pp. 381–9. ISSN: 1044-677X (Print) 2165-7254 (Electronic) 1044-677X (Linking). doi: [10.6028/jres.106.015](https://doi.org/10.6028/jres.106.015).
- [281] C. Anasooya Shaji, B. D. Robinson, A. Yeager, M. R. Beeram, M. L. Davis, C. L. Isbell, J. H. Huang, and B. Tharakan. "The Tri-phasic Role of Hydrogen Peroxide in Blood-Brain Barrier Endothelial cells". In: *Sci Rep* 9.1 (2019), p. 133. ISSN: 2045-2322 (Electronic) 2045-2322 (Linking). doi: [10.1038/s41598-018-36769-3](https://doi.org/10.1038/s41598-018-36769-3).
- [282] M. S. Adil and P. R. Somanath. "Endothelial Permeability Assays In Vitro". In: *Methods Mol Biol* 2367 (2021), pp. 177–191. ISSN: 1940-6029 (Electronic) 1064-3745 (Linking). doi: [10.1007/978-1-0705-2020-309](https://doi.org/10.1007/978-1-0705-2020-309).
- [283] R. Bednarek. "In Vitro Methods for Measuring the Permeability of Cell Monolayers". In: *Methods Protoc* 5.1 (2022). ISSN: 2409-9279 (Electronic) 2409-9279 (Linking). doi: [10.3390/mps5010017](https://doi.org/10.3390/mps5010017).
- [284] S. Faber and S. McCullough. *FITC-Dextran Trans-Epithelial permeability assay*. Report. 2020. doi: <https://doi.org/10.21203/rs.2.20495/v1>.
- [285] S. B., K. A.R, E. M.B, A. H.E, S. M.L, and H. J.J. "TEER Measurement Techniques for In Vitro Barrier Model Systems". In: *SLAS Technology* 20 (2015), pp. 107–126. ISSN: 2472-6303. doi: <https://doi.org/10.1177/2211068214561025>.
- [286] G. van Meer and K. Simons. "The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells". In: *EMBO J* 5.7 (1986), pp. 1455–64. ISSN: 0261-4189 (Print) 1460-2075 (Electronic) 0261-4189 (Linking). doi: [10.1002/j.1460-2075.1986.tb04382.x](https://doi.org/10.1002/j.1460-2075.1986.tb04382.x).
- [287] M. Cereijido, E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini. "Polarized monolayers formed by epithelial cells on a permeable and translucent support". In: *J Cell Biol* 77.3 (1978), pp. 853–80. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). doi: [10.1083/jcb.77.3.853](https://doi.org/10.1083/jcb.77.3.853).
- [288] Corning and Life Sciences. *Permeable supports - Product selection guide*. Catalog. 2021.
- [289] J. M. May, Z. C. Qu, and R. R. Whitesell. "Generation of oxidant stress in cultured endothelial cells by methylene blue: protective effects of glucose and ascorbic acid". In: *Biochem Pharmacol* 66.5 (2003), pp. 777–84. ISSN: 0006-2952 (Print) 0006-2952 (Linking). doi: [10.1016/s0006-2952\(03\)00408-8](https://doi.org/10.1016/s0006-2952(03)00408-8).
- [290] J. Bicas, D. Kleinegris, and M. Barbosa. "Use of Methylene Blue uptake for assessing cell viability of colony-forming microalgae". In: *Alga Research* 8 (2015), pp. 174–180. doi: <https://doi.org/10.1016/j.algal.2015.02.004>.
- [291] M. Kwolek-Mirek and R. Zadrag-Tecza. "Comparison of methods used for assessing the viability and vitality of yeast cells". In: *FEMS Yeast Res* 14.7 (2014), pp. 1068–79. ISSN: 1567-1364 (Electronic) 1567-1356 (Linking). doi: [10.1111/1567-1364.12202](https://doi.org/10.1111/1567-1364.12202).
- [292] N. Mandal, E. Monfort-Sanchez, J. Avery, et al. "A wireless semi-wearable sensor for assessment of gut function in low-resource setting". In: *SPIE Proceedings, Optics and Biophotonics in Low-resource Setting XI, Wearable and related technologies* (2024). doi: [12832-8](https://doi.org/10.1117/1.567-1364.12202).
- [293] L. Sun, J. Ouyang, F. Zeng, and S. Wu. "An AIEgen-based oral-administration nanosystem for detection and therapy of ulcerative colitis via 3D-MSOT/NIR-II fluorescent imaging and inhibiting NLRP3 inflammasome". In: *Biomaterials* 283 (2022), p. 121468. ISSN: 1878-5905 (Electronic) 0142-9612 (Linking). doi: [10.1016/j.biomaterials.2022.121468](https://doi.org/10.1016/j.biomaterials.2022.121468).

- [294] R. Wang, L. Zhou, W. Wang, X. Li, and F. Zhang. "In vivo gastrointestinal drug-release monitoring through second near-infrared window fluorescent bioimaging with orally delivered microcarriers". In: *Nat Commun* 8 (2017), p. 14702. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: [10.1038/ncomms14702](https://doi.org/10.1038/ncomms14702).