UNIVERSITY OF BIRMINGHAM

EXPLORING THE PAEDIATRIC GASTROINTESTINAL ANATOMY BY GENERATING NOVEL DATA ON FLUID VOLUMES, LOCALISATION AND COMPOSITION

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I would like to dedicate this thesis to my new-born twins, Mary-Ann and Asimenia, hoping that as they grow up they will be proud for their mother, as I am already proud for them for being little fighters, and that they will understand that they should stop at nothing when they chase their dreams.

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Glossary

API (Active Pharmaceutical Ingredient)
FaSSCoF (Fasted State Simulated Colonic Fluid)

FaSSGF (Fasted State Simulated Gastric Fluid),
FaSSIF (Fasted State Simulated Intestinal Fluid)
FeSSCoF (Fed State Simulated Colonic Fluid)
FeSSIF (Fed State Simulated Intestinal Fluid)
GI (Gastro-intestinal tract)
HIF (Human Intestinal Fluid)
IRAS (Integrated Research Approval System)
LLQ (Left Lower Quadrant)
LUQ (Left Upper Quadrant)
MRC (Medical Research Council)
MRI (Magnetic Resonance Imaging)
NHS (National Health System)
NRES (National Research Ethics Service)
PBPK modelling (Physiologically Base Pharmacokinetic modelling)
M & S (Modelling and Simulation)
PD (Pharmacodynamics)
RLQ (Right Lower Quadrant)
RUQ (Right Upper Quadrant)
SIF (Simulated Intestinal Fluid)
UoB (University of Birmingham)

Abstract

A novel method for magnetic resonance image (MRI) analysis was developed to measure fluid volumes in paediatric GI images. A combination of the HOROS and ImageJ softwares was used. The method was able to i) identify the water content in the paediatric gastrointestinal (GI) tract and to ii) quantify its volume. Data from 32 fasted children (aged 0-16 years) and 23 fluid-fed (aged 8-16 years) was evaluated. Previous studies that included adult populations showed that water in the GI tract does not exist in a continuous form but creates “fluid pockets”. The mean volume of fluid pockets in the small intestine was 7.4 ml for the fasted state and 30.4 ml for the fluid fed state. This study demonstrated that the water content has a discontinuous pattern in the form of fluid pockets for paediatric population as well. The location and the volume of the paediatric GI fluid pockets can have a significant impact on paediatric oral drug absorption.

These novel paediatric GI data have been input into the physiologically based pharmacokinetic model (PBPK) model SimCyp, in an attempt to update the software and its current paediatric default mode.

Additionally, characterisation of simulated gastric and intestinal fluids took place. Characterisation methods used for human samples analysis were developed by working on

simulated gastric and intestinal fluids. In total, 55 children have participated aged 11 months to 15 years. From them, 53 gastric fluid samples and 40 intestinal samples were obtained. The human samples were characterised for their pH, buffer capacity and osmolality. It was observed that gastric osmolality ranged from 1-615 mOsm/kg, while the intestinal osmolality ranged from 35-631 mOsm/kg. the median pH values were 2.5 for gastric fluids and 3.27 for intestinal fluids. The buffer capacity did not change significantly as both gastric and intestinal buffer capacity values displayed medial values of 12 mM/L/ΔpH.

A literature search was conducted to identify the bibliography describing how the paediatric gastrointestinal physiology and anatomy changes in malnourished children. The narrative review demonstrated that several differences in the GI anatomy and physiology, including reduced saliva secretion, increased gastric pH, slower gastric emptying, increased levels of bacteria in the small intestine, reduced surface area of intestinal villi and increased intestinal permeability. Many of these data were older than 30 years and included heterogeneous population; therefore, future work should focus on generating new, updated data on the impact of malnutrition on the paediatric GI tract, and subsequently on oral drug absorption.

1. Chapter 1: Introduction

1.1 Thesis Aims and Objectives

The aims of this thesis were to generate knowledge of paediatric gastrointestinal volumes and gastrointestinal fluid composition to better understand and model drug absorption. The complex paediatric physiology and the limited pharmacokinetic data that are available, have made understanding paediatric oral drug absorption challenging. Additionally, the ethical restraints associated with paediatric clinical trials has limited generation of relevant knowledge.

1.2 First part of thesis:

The objectives of the first part of the thesis were:

- To measure the mean and range of fluid volumes within the stomach and small intestine of children.
- To quantify the mean and range of number of fluid pockets distributed within the GI tract.

This research was the first study to calculate paediatric gastrointestinal (GI) volumes and identify fluid localisation within the GI tract through a novel MRI method development.

1.3 Second part of the thesis:

The second part of the thesis, focused on characterising paediatric GI samples from the stomach and duodenum for their pH, osmolality and buffer capacity. This was done in accordance with the aim of the thesis, which has been to generate knowledge on the paediatric GI tract.

The objective of the second part of the thesis was:

- To characterise the properties of gastric and small intestinal fluids from children in terms of: pH, buffer capacity, osmolality, and the qualitative and quantitative composition of bile salts.

All this information would be ultimately used to update the existing paediatric simulated fluids and allow thus mimicry of the paediatric GI composition.

Additionally, one theme of the thesis was to discuss malnutrition and its impact on the paediatric GI tract. Also, it was studied and discussed how these changes subsequently affect oral drug absorption in the paediatric malnourished population.

1.4 Thesis Breakdown

This thesis is structured in the following manner:

Chapter 1: Introduction gives an overview of the research conducted for the thesis, its aims and objectives. It also lists the publications, together with the presentations that have arisen from the thesis.

Chapter 2: Background Chapter provides an overview of the published literature on the GI environment with a focus on how this environment affects oral drug absorption. It explores the GI conditions which are critical to oral drug absorption, as well as the impact of food on the luminal environment. The chapter reviews adult and paediatric GI anatomy in an attempt to identify similarities and differences. The exploration of the adult GI tract informs the research approach towards and understanding of the complex paediatric GI tract, which, in turn, aids the identification of gaps in the literature surrounding the paediatric GI tract. In this way, up-to-date knowledge on paediatric simulated fluids is provided to allow mimicry of paediatric intestinal composition.

Chapter 3: Malnutrition chapter is my joint publication with the University of Greifswald and Janssen, which describes the impact of malnutrition on absorption from the paediatric GI tract.

Chapter 4: Magnetic Resonance Images (MRI) analysis chapter is my published study, which describes the development of a novel MRI method of analysis to identify and quantify the fluid volume in the paediatric GI tract (stomach and small intestine). In the past, it has been shown that fluid exists in discontinuous fluid pockets in adults [Schiller *et al*., 2005]. The novel MRI method reported in this chapter aimed to find out if this happens for paediatric patients as well. This is the first published study to analyse MRI images from paediatric patients and report fluid pocket identification and localisation.

Chapter 5: Physiological Based Pharmacokinetic Model (PBPK) Model uses SimCYP paediatric physiologically based pharmacokinetic modelling (PBPK) software and incorporates the fluid

volumes identified from Chapter 4: Magnetic Resonance Images (MRI) analysis chapter to model the pharmacokinetics of ritonavir in children.

6. Chapter 6: Simulated Fluids chapter presents the work done on characterising simulated intestinal fluids (SIFs) in order to develop methodologies and techniques to characterise the GI samples in

7. Chapter 7: Gastro-intestinal human samples analysis chapter

7. Chapter 7: Gastro-intestinal human samples analysis chapter is my joint publication with Birmingham Children's Hospital and Janssen, which describes the analytical method

development and characterisation of gastric and duodenal intestinal fluid samples collected from children at Birmingham Children's Hospital (BCH). The samples have been characterized to determine their pH, osmolality, buffer capacity and bile salt composition and concentration.

8. Chapter 8: Conclusion is the final chapter of the thesis, discussing the outcomes, the limitations and the room for research that exists in this field.

1.5 Publications arising from thesis

- Pawar, G., Papadatou Soulou, E., Mason, J., Muhammed, R., Watson, A., Cotter C., Abdallah, M, A., Harrad, S., Mackie, C., Arien, T., Inghelbrecht, S., Batchelor, H. (2021). Characterisation of fasted state gastric and intestinal fluids collected from children. *European journal of Pharmaceutics and Biopharmaceutics*. 158, 156-165.

- Papadatou-Soulou, E., Mason, J., Parsons, C., Oates, A., Thyagarajan, M. and Batchelor, H. (2019). Magnetic Resonance Imaging Quantification of Gastrointestinal Liquid Volumes and Distribution in the Gastrointestinal Tract of Children. *Molecular Pharmaceutics*, 16(9), 3896-3903.

- Freerks, L., Papadatou Soulou, E., Batchelor, H. and Klein, S. (2019). A review of GI conditions critical to oral drug absorption in malnourished children. *European Journal of Pharmaceutics and Biopharmaceutics*, 137, 9-22.

1.6 Conference presentations arising from thesis

- 12th European Paediatric Formulation Initiative (EuPFI), virtual conference 2020. "Characterisation of Fasted State Paediatric Gastrointestinal samples" [Poster Presentation]

- 11th European Paediatric Formulation Initiative (EuPFI), Malmo, Sweden 2019. "The impact of using relevant luminal fluid volumes on a PBPK model of Ritonavir absorption in children". [Poster Presentation]
  Published online: Papadatou Soulou, E., Pawar, G., Inghelbrecht, S., Mackie, C., Arien, T. and Batchelor, H., 2019. *The Impact Of Using Relevant Luminal Fluid Volumes On A PBPK Model Of Ritonavir Absorption In Children</P>*. [online] F1000research.com. Available at: <https://f1000research.com/posters/8-1689> [Accessed 1 April 2020].

  Awarded by the 11th EuPFI committee: Young Scientist Travel Award, 2019.

- 10th European Paediatric Formulation Initiative (EuPFI), London, UK 2018, United Kingdom: "Quantification and determination of the water content in the GI tract through analysis of small bowel MRI".
  [Oral and Poster Presentation]

2. Chapter 2: Background Chapter

The gastrointestinal (GI) environment can have an impact on the pharmacokinetics (PK) of orally administered drug formulations and subsequently, cause suboptimal efficacy and therapeutic outcomes. The absorbable dose is influenced by the transit time, concentration of active substance at the absorbing tissue, the available surface area and the permeability rate of the drug across the membrane. It is expected, that any differences in these parameters can potentially affect the percentage of the absorbed/ required drug in the body. Until now, most studies have focused on adults and results subsequently extrapolated to children. A greater understanding of the actual conditions in the GI environment will enable more realistic predictions of drug absorption in paediatric populations.

The majority of drugs are administered orally to children [Nicolas *et al*., 2017]. Therefore, the factors affecting paediatric intestinal absorption must be investigated. Intestinal absorption depends on drug characteristics (e.g. physicochemical properties), physicochemical parameters (e.g. gastrointestinal fluid composition, volume, transit time, microbiota, drug metabolizing enzymes, drug transporters) and environmental factors (e.g. food, drug formulation). It is recognised that these factors vary with age as different paediatric ages are characterised by unique developmental characteristics [Nicolas *et al.,* 2017]. Therefore, it is essential that paediatric patients are divided into age groups to be explored accordingly. The International Conference on Harmonization (ICH) E11 has classified the paediatric age groups as following: new-born (0–28 days), infant (>28 days–12 months), toddler (>12 months–23 months), preschool child (2–5 years), school age child (6–11 years), adolescents (12–18 years).

Oral drugs can be administered as solid dosage forms e.g tablets and capsules. Prior to absorption, disintegration and dissolution are essential steps [Kimura and Higaki, 2002]. For suspensions dissolution can be a rate limiting step prior to absorption.

The focus of this thesis is oral absorption of drugs in children. This chapter reviews the similarities and differences in the GI anatomy of children when compared to adults. The next sections focus on gastric and small intestinal anatomy in both adults and children.

2.1 Stomach

Prior to absorption from the small intestine, orally administered drugs pass through stomach. Subsequently, the residence of a dosage form in the gastric environment, may influence drug absorption from the gastrointestinal tract [Van den Abeele *et al*., 2018]. The stomach lies on the left upper quadrant of the abdomen [Gray, H., 2012]. It has five main anatomical features: cardi, fundus, body, antrum and pylorus. Cardia is the first part after the oesophagus and it contains the cardiac sphincter, which prevents gastric contents of going back to the oesophagus. The rounded area to the left of the cardia and just below the diaphragm is called "fundus". The body is considered is the biggest and most major compartment of the gastric area. Its role is pivotal as it is where the food is mixed and the decomposition of it starts. The lower part of the stomach is called antrum, and its function is to hold the decomposed food until it passes to the small intestinal tube. The pylorus is the sphincter that connects the stomach to the small intestine, therefore it plays a crucial role on passing on the active pharmaceutical ingredients (API)s and/ or nutrients from food to the small intestine for absorption. Figure 2 1 illustrates the gastric anatomy.

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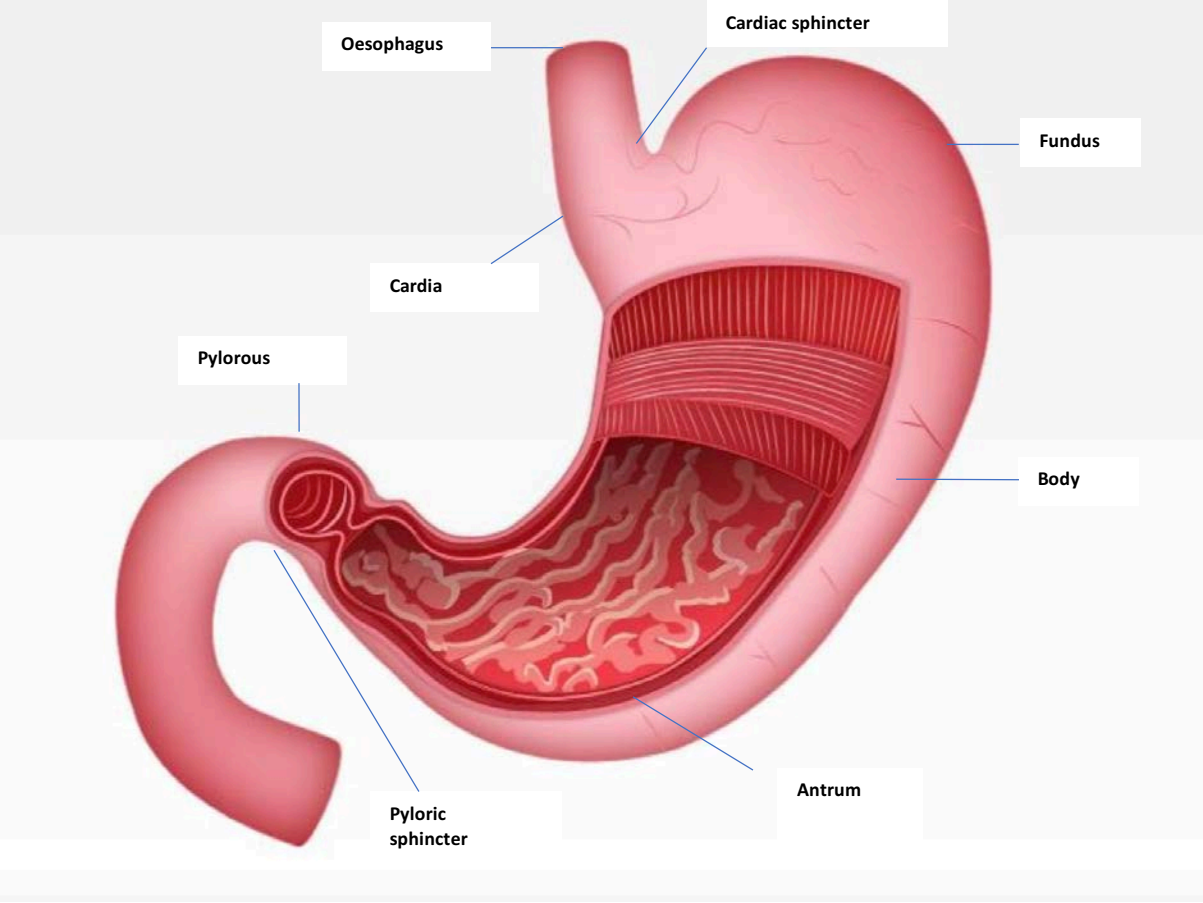


Figure 2 1 The stomach Anatomy (Figure adapted from Slideteam.net [2021].

2.1.1 Gastric anatomy in children

In children, the stomach is small at birth. However, the organ increases to approximately 5 times its initial size in the first few days, after the swallowing and feeding process has commenced. Acid secretion, which is the ability of parietal cells to secrete hydrochloric acid and is stimulated by food consumption, starts in the first days of the neonate's life and gastric size increases from only about 6 ml at birth to 30 ml in the first days of its life, to about 100 ml in the 4th week of the baby's life [Lander and Newman, 2010].

2.2 Small intestine

The dominant site for drug absorption is the small intestine [Murakami, 2017] Its higher pH creates an environment that promotes the dissolution of acidic compounds. Poorly soluble weak bases are expected to have a higher solubility in the gastric environment compared to the small intestine [Mann *et al*., 2007]. In adults, the small intestine extends approximately to 6 meters in length, while its diameter is about 2.5-3 cm. Adult normal jejunal and ileal wall thickness is less than 3 mm, with the jejunum having a slightly larger diameter compared to

the ileum [Jones *et al*., 2019]. The duodenum is the first part of the small intestine, proximal to the stomach. The duodenum is small in size in relation to the other parts forming the small intestine. The duodenum is characterised by its C shape and it is approximately 25 to 30 cm long in an adult small intestine. Its name "duodenum" in Latin means 12 fingers, which is about the actual length of the duodenum [Lopez et al., 2020]. The jejunum, in the central abdomen, holds approximately 2/5 of the rest of the GI tract. Interestingly, its colour is deep red, which is explained by the extensive blood supply of the area. The ileum, the last section of the small intestine, holds the remaining 3/5 of the GI tract [Britannica, 2018].
The colon, which is the continuation of the small intestine, is about 1.5 m length in adults, with a diameter of 6-7.5 cm.

The whole adult GI tract system is responsible for handling 8-10 L daily of fluid, which contains about 800 mmol Na, 700 mmol Cl and 100 mmol K. While the majority of the absorption takes place in the small intestine, and more specifically in the jejunum, an approximate amount of 1.5 L is left to the large bowel to absorb. A volume of about 100 ml is lost daily through the stools [Kiela and Ghisan, 2016].

Villi, which in Latin means "shaggy hair", are tiny finger like pieces of tissue and are about 0.5-1.6 mm in length. Each villus has many microvilli on the epithelial surface, forming a brush border. Microvilli are found at the surface of plenty cell types, but they exist in abundance on simple epithelial, as for example on intestinal mucosa and the surface of kidney proximal tubule [Friederich and Louvard, 2006]. The main function of both villi and microvilli is to increase the surface area intended for absorption and to increase small intestinal absorption. Interestingly, villi and microvilli presence increase the intestinal surface area by 30- 600- fold, respectively [Kiela and Ghisan, 2016]. Figure 2 2 demonstrates microscopically villi and microvilli.

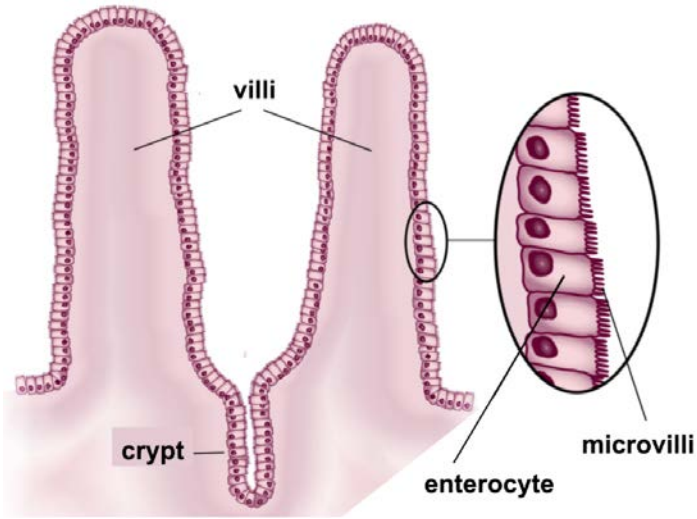


Figure 2 2 Illustration of villi and microvilli.
By Ballena Blanca (CC BY-SA 4.0) via Commons Wikimedia [2021]

Each villus is composed of a network of capillaries and thin lymphatic vessels called *lacteals*, which are located close to its surface. Nutrients and drugs are transferred by the epithelial cells of villi from the intestinal lumen into these capillaries. Figure 2 3 is an illustration of the structure of the intestinal villus, while Figure 2 4 demonstrates how a section of duodenum is organised and the parts that constitute it.

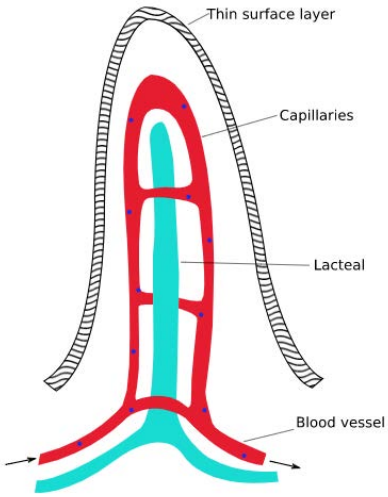


Figure 2 3 Intestinal villus. A scheme of the structure of the villus. Thin surface layer appears above the capillaries, which are connected to the blood vessel. Capillaries surround the lacteal. Provided via Commons Wikimedia. License: Public domain: no known copyright.

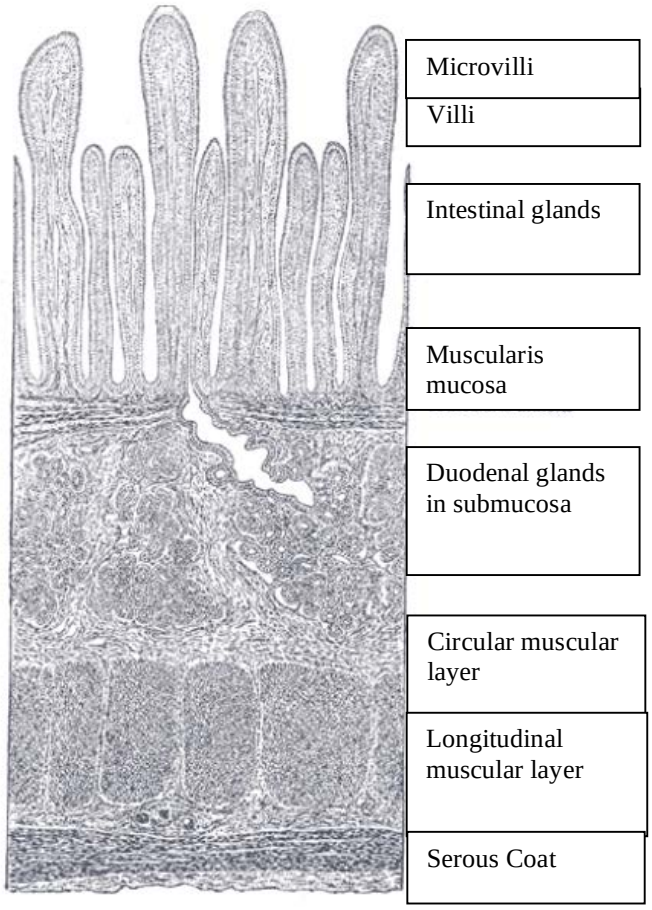


Figure 2 4 Section of duodenum with villi at the top layer, demonstrating all the parts that constitute it, including from the bottom to the top: serous coat, longitudinal muscular layer, circular muscular layer, duodenal glands in submucosa, muscularis mucosa, intestinal glands, villi. Figure adapted from Gray and Carter [1918].
Public Domain, No known copyright.

2.2.1 Small intestinal anatomy in children

Until the paediatric small intestine is fully developed and has acquired adult characteristics and dimensions, it has fewer and less marked circular folds than adults (Lander and Newman, 2010). Its length in a term baby is about 300- 350 cm, under gentle tension and the mesentery removed [Lander and Newman, 2010]. Additionally, the paediatric small intestine lies in a more transverse orientation than in adults, because of the anatomy of their abdominal bladder [Lander and Newman, 2010]. Although the paediatric abdominal bladder is considered intra-abdominal, unlike adults, only half of it is located within the pelvic cavity. It becomes entirely pelvic approximately in the 6th year of life of the child [Lander and Newman, 2010].

As the small intestine develops, it increases in length approximately 20 times before reaching the 6 meters length of an adult [Weaver *et al.,* 1991].

Previous evidence suggests that numerous physiological factors (e.g. gastro-intestinal pH, volume, transit time, morphology) and biochemical factors (e.g. luminal enzymes and flora, intestinal enzymes and transporters) can have significant differences between children and adults [Nicolas *et al*., 2017]. These differences could lead to age-related changes in drug exposure and response; the different physiological factors potentially affecting drug absorption. Drug dosage adjustment is, therefore, likely to be essential for paediatric patients to ensure optimal therapeutic outcomes and minimise potential side effects [Nicolas *et al.,* 2017]. An exemplary illustration of unwanted toxicity due to false dosing deriving from lack in paediatric data is the gray baby syndrome in neonates receiving chloramphenicol [Sutherland, 1959]. Neonates, especially pre-term ones born earlier than the 37th week of gestation, began to develop the following symptoms within two days of birth when given chloramphenicol: abdominal distention, vomiting, hypothermia, cyanosis, and cardiovascular instability. This is due to that they are receiving a very high dose, which has been inaccurately scaled from an adult estimation. Vasomotor collapse causes mottling of the skin, and subsequently, an ashen- gray skin discolouration named the reaction “gray-baby syndrome” [Cumming *et al.,* 2020].

Despite the fact that huge progress has been made in exploring the paediatric gastro-intestinal physiology thanks to the co-ordinated efforts from the pharmaceutical industry, academics and regulatory bodies, there are still knowledge gaps that have to be filled [Lennernas *et al.,* 2014].

This thesis will explore the knowledge gaps surrounding paediatric GIT physiology (Chapter 4: Magnetic Resonance Images (MRI) analysis chapter and

7. Chapter 7: Gastro-intestinal human samples analysis chapter). In order to provide context for the knowledge gaps presented, the next sections will summarise the process of dissolution and bioavailability for oral medicines and the impact of food.

2.3 Drug Dissolution and Bioavailability

Drug dissolution in the GI tract is the first step taking place prior to oral absorption of a solid orally administered active pharmaceutical ingredient (API). It is fundamental that only the dissolved drug can permeate the small intestinal mucosa at the absorptive areas [Dressman *et al*., 2000]. Otherwise, the therapeutic effects may not be the optimal. Thus, a drug's solubility and dissolution are both crucial for its therapeutic potential, where systemic absorption is the target. The volume and composition of the fluid available will dictate both the solubility and dissolution of a drug administered orally hence knowledge of these factors is critical in predicting bioavailability.

Food will also alter bioavailability. When evaluating the impact of food impact, composition and timing of consumption are the two major parameters that have to be taken into consideration. Current regulatory bodies' recommendations to maximise the therapeutic effect of drugs in adults suggest the use of high calorie, high fat (approximately 50% of total content) meals that consist of 150, 250 and 500-600 calories of protein, carbohydrates and fat respectively [FDA, 2010; EMA, 2010].

An adult reference meal has been described as two fried eggs in butter, two strips of bacon, two slices of toast with butter, four ounces of hash brown potatoes and glass of whole milk. Regulatory bodies also suggest that drug administration should be approximately half an hour after meal initiation with a water glass [FDA, 2010; EMA, 2010].

2.4. Gastric volume in the fed state

For adults, the volume of gastric contents after consumption of a solid meal containing half the calories of the reference meal is reported to be as similar to the total volume of the meal (about 400 ml) for the first hour post-eating [Pentafragka *et al*., 2018]. Then, approximately four hours after a meal, volume numbers return to baseline numbers [Malagelada *et al*., 1976].

According to MRI data [Koziolek *et al*., 2014], intra-gastric volumes increase to 580 ml in 15 minutes following consumption of the reference meal in adult subjects. This observed increase is approximately 100 ml over the volume of the reference meal (about 480 ml) [Klein *et al*., 2004].

2.5 Small intestinal volume in the fed state

Both direct sampling and imaging techniques have been used for extracting information on the small intestinal environment in the fed state in adults. However, the nature of extracted information is different across these techniques. Modelling of intestinal adult data, has estimated the volume of adult duodenal contents to be approximately 30 ml in the fasted state 1-hour post administration of a non-caloric 240 ml aqueous solution [Kourentas *et al*., 2016]. Data from healthy adults collected by aspiration, show that as soon as someone starts eating a solid meal of 645 ml and for up to 2 hours later the volume of the duodenum and jejunum contents were 1500 ml and 750 ml respectively [Fordtran *et al*., 1966].

MRI data show that food intake decreases the mean fluid volumes in the whole small intestine from approximately 100 ml to 50 ml in the fasted state one-hour post meal consumption [Schiller *et al*., 2005]. It has been suggested that more studies are needed to understand the impact of meal on the volume of contents in the upper small intestine, as different volumes and compositions may have a significant effect on oral drug absorption [Pentafragka *et al*, 2018].

2.6 Fluid pockets in the GI tract and their importance for oral drug absorption

Fluid pockets in the GI tract were first reported in a study conducted by Schiller *et al*., [2005], using an MRI technique. The study included adult subjects and demonstrated that intestinal fluid is located in pockets of variable volume, irregularly distributed across the GI tract. It was suggested that the inhomogeneous distribution of fluid in the GI tract possibly contributes to increased variability of drug release, which lead to differences in drug absorption [Schiller *et al*., 2005]. Then, further research conducted by Mudie *et al* [2014], also with adult subjects, quantified total volume and distribution of fluid in the GI tract. Mudie's study [2014] confirmed the Schiller *et al*. [2005] findings that discontinuous fluid pockets exist in the small intestinal tract. Murray *et al* [2017] further verified the existence of fluid pockets in the colonic environment in adult subjects. My study [Papadatou Soulou *et al*., 2019] aimed to explore whether water exists in a discontinuous pattern in paediatric subjects similar to that seen in adults. MR images were collected from children undergoing MR scanning as part of routine clinical practice at BCH. In order to explore if water exists in fluid pockets across the

paediatric GI tract a novel MRI method has been developed and is further explained at chapter Chapter 4: Magnetic Resonance Images (MRI) analysis chapter.

2.7 PBPK models for oral drug absorption

Physiologically based pharmacokinetic models assist researchers in predicting pharmacokinetics as accurately as possible. To create a robust PBPK model, all the parameters that can potentially impact the drug absorption, distribution, metabolism and elimination should be identified and then incorporated into the PBPK model [Barrett *et al*., 2012].

Although PBPK software models were primarily built on data from adult subjects in order to predict drug absorption from the adult body, paediatric modules have been added as the physiological differences between adults and children have been identified. In the past, the drug dosage for paediatric patients has been calculated from adult data, using "allometric scaling" [Baber and Pritchard, 2003]. These modules, include mathematical terms that describe the relationships between age and the developmental ages in the body, including: body weight, body height, organ volume, cardiac output, renal function, plasma protein binding and some key drug metabolizing enzymes (e.g. CYPs) [Johnson *et al.,* 2006; Parrott *et al.,* 2011; Edginton, 2011]. Paediatric PBPK models also include mathematical formulas that cover the developmental changes that occur in the GI tract e.g. age-related changes in organ volume, absorptive surface area, permeability, enzymes activity, pH, intestinal fluid content and transit time [Vertzoni *et al*., 2010].

Today, PBPK modelling software is widely used (e.g. Simcyp®, PKSim®, Gastroplus) [Bouzom *et al*., 2012]. Among several PBPK software options, the SimCyp Population based Simulator has been established as the preferred option of pharmaceutical companies [Jamei *et al.,* 2013b]. One of the most recent versions of this software is SimCyp paediatric software v.18. The MRI data from my publication Papadatou Soulou *et al.* [2019] (Chapter 4: Magnetic Resonance Images (MRI) analysis chapter) suggest that the intestinal volumes for paediatric fasted and fed paediatric populations should be updated in the current PBPK software. In Chapter 5: Physiological Based Pharmacokinetic Model (PBPK) Model of this thesis, the intestinal fluid volume data from this (Chapter 4: Magnetic Resonance Images (MRI) analysis chapter have been incorporated into SimCyp paediatric model to explore the absorption of

ritonavir as model drug.

The new updated model has been presented in Chapter 5: Physiological Based Pharmacokinetic Model (PBPK) Model and compared with the default SimCyp model.

2.8 Physiochemical properties of Human Intestinal fluids and their impact on oral drug absorption

GI fluid is characterised by high inter- individual variability. The majority of studies so far have included adult subjects and characterised GI fluids from the fasted and fed state [Lindahl *et al.,* 1997; Pedersen *et al.,* 2000; Persson *et al.,* 2005; Clarysse *et al*., 2009; Heikkila *et al*., 2011; Perez de la Cruz Moreno *et al*., 2006; Foltz *et al*., 2015; Holmstock *et al.,* 2013; Riethorst *et al.,* 2016; Pentafragka *et al.,* 2019; Vertzoni *et al.,* 2012].

In order to predict the *in vivo* performance, simulated intestinal fluids (SIFs), based on the composition of aspirated intestinal fluids, have been widely used. This method has shown superiority compared to simple buffers in terms of predicting *in vivo* performance [Mann *et al*., 2017].

2.9 Simulated Gastric and Intestinal fluids

Despite the fact that the use of human intestinal fluids (HIFs) have been described as a gold standard [Soderling *et al*., 2010], their use is extremely challenging and in most cases, not feasible due to ethical limitations and restricted availability. Therefore, SIFs have been created as an alternative [Klein, 2010]. SIFs are being continuously updated to predict as close as possibly the oral absorption of drugs. The pre- and post- prandrial state are reflected by different compositions of SIFs to study and understand different food scenarios. SIFs' compositions so far aimed not only to reflect pH, buffer capacity and osmolality values in HIFs, but also depict the bile salts' and phospholipids' concentration.

Several research groups have aimed to reflect the entire human GI tract with SIFs, creating: FaSSGF (Fasted State Simulated Gastric Fluid), FaSSIF (Fasted State Simulated Intestinal Fluid) and FeSSIF (Fed State Simulated Intestinal Fluid) and FaSSCoF (Fasted State Simulated Colonic Fluid) and FeSSCoF (Fed State Simulated Colonic Fluid) [Galia *et al*., 1998; Vertzoni *et al.,* 2005; Jantratid *et al.,* 2008; Vertzoni *et al*., 2010]. Their continual update has been crucial in improving the accuracy of the prediction of oral drug absorption.

Dressman *et al.* [1998] and Galia *et al.* [1998] developed FaSSIF/ FeSSIF on adult data. The composition of these SIFs included pH, buffer capacity, osmolality, and bile salts to reflect the actual parameters of the HIFs and to increase the media's biorelevance. Next, Jantrid *et al.* [2008] updated the compositions by developing FaSSIF-V2 and FeSSIF-V2. Jantrid *et al.* [2008] achieve an enhanced stability. Fuchs *et al* in 2014 further developed the formulation, FaSSIF-V3. Furthermore, different versions of FaSSIF-V3 have been created in an attempt to realistically reflect the concentrations of different bile salts [Fuchs *et al.,* 2014]. It has been shown that FaSSIF- V3- GC/ TC Chol was the ideal version among others to represent as close as possible the solubility of the most drugs in fasted HIF.

6. Chapter 6: Simulated Fluids chapter of this thesis describes the optimisation of characterisation techniques with SIF for use with HIF samples obtained from Birmingham Children's Hospital (BCH) and collected with endoscopy as a part of their clinical routine.

The next paragraphs provide an insight into the key physicochemical properties of the gastric and intestinal environment in children.

2.10. Paediatric Gastrointestinal fluid composition

2.10.1 Gastric emptying

Gastric emptying (GE) in adults is biphasic with an initial rapid phase (<10 minutes), which is followed by a slower emptying [Ziessman *et al*., 1992]. Evidence demonstrates that infants' GE below 6-8 months is slower compared to adult values, due to the immature motility of neuro-regulation [Nicolas *et al*., 2017].

2.10.2 Gastric volume

Gastric volume is another key parameter that influences oral drug solubility and subsequently, intestinal absorption. After normalisation per kg body bodyweight, fasted gastric volumes are reported to be broadly similar for both adults and children: 0.36-0.50 ml/kg [Mudie *et al*., 2014; Goetze., 2009] and 0.25- 0.56 ml/kg respectively [Schwartz *et al*., 1998; Crawford *et al*., 1990; Meakin, 1987], while the drug dosage may be different.

2.10.3 pH of paediatric gastric and intestinal fluids

Gastric pH values are in a neutral range (pH= 6-8) in new born babies, a fact probably attributed to the amniotic fluid in the stomach [Avery *et al.,* 1966]. However, contradictory data reports that in the first couple of hours after birth, a decline in pH has been reported, which is then followed by a rise to neutral pH after 24-72 hours [Sager *et al.,* 2014; Bowles *et al.,* 2010; Batchelor and Marriott, 2015; Milsan and Jusko, 1994; Bartelink et al., 2006; Koren, 1997]. Next, a progressive decrease in the pH has been reported from infancy to adulthood, which finally leads to the adults' acidic values [Sage *et al*., 2014; Bowles *et al*., 2010; Milsap and Jusko, 1994; Bartelink *et al*., 2006; Koren, 1997]. As mentioned, there is opposing evidence showing that the gastric pH remains stable around acidic values less than 3, from preterm neonate until adulthood [Nicolas *et al*., 2017].

Although there are several studies reporting paediatric intestinal pH, the data are conflicting, as the intestinal pH values range from 4.5-12, and their intestinal mean or median pH values range from 6-8. Furthermore, they represent various age groups [Krafte- Jacobs *et al.,* 1996; Gharpure *et al*., 2000; Westhus *et al.,* 2004; Metheny *et al.,* 1999]

2.10.4 Buffer Capacity of paediatric gastric and intestinal fluids

The fact that no published literature exists that report the buffer capacity in paediatric HIFs is of great interest. Previous studies have reported that buffer capacity for fasted adult HIFs range from 2.5-13 mM/L/ΔpH [Fuchs *et al*., 2014; Kalantzi *et al.,* 2006]. Higher buffer capacity values are described in the fed state and post-water consumption [Kalantzi *et al.,* 2006]. Today, the proposed buffer capacity for paediatric SIFs is 10 mM/L/ΔpH [Maharaj *et al*., 2016].

2.10.5 Osmolality of paediatric gastric and intestinal fluids

At the moment, the information on osmolality in paediatric populations is very limited. Previous studies on aspirated gastric fluids include mean values of 253 mOsm/L, 274 mOsm/kg, 188 mOsm/kg and 219 mOsm/kg, for a sample of children under 2 years of age, for neonates, for children aged 2-12 years old and for adolescents respectively [Wakayama et al., 1998; Van den Abeele et al., 2018]. Although some studies report osmolarity (per liter) and other osmolality (per kg), the units can be used the same way if it is hypothesized that carrier's fluid density is 1 kg/L. The current paediatric fasted state simulated gastric fluid osmolality value is 120.7 mOsm/kg [Majaraj *et al.,* 2016]. On the other hand, the osmolality value of paediatric fasted state simulated intestinal fluid is 180 mOsm/kg. It is essential to acknowledge that the presence of food may have a significant impact on the osmolality of gastric fluids [Van den Abeele *et al*., 2018].

2.10.6 Bile salts in the paediatric gastric and intestinal fluids

Bile acids are steroid acids, which when are conjugated with taurine or glycine, provide bile salts. Bile salts are amphipathic molecules, that are created from cholesterol in the liver [Yang *et al.*, 2011]. Their synthesis is the main route for reducing the cholesterol levels in the body (action is crucial as they act as absorption enhancers to increase the drug transport across several biological barriers (blood brain barrier, mucosa, skin etc.) [Yang *et al.,* 2011]. They act so, by enhancing the solubility of poorly soluble drugs [Coufalova *et al*., 2013]. Bile salts are divided to primary bile salts (cholate and chenodeoxycholate), which after bacterial action form the secondary bile salts. Maharaj *et al*. [2016] reported a bile salt concentration of 0.02 mM for neonates and 0.06 mM for infants for paediatric fasted state simulated gastric fluids. Interestingly, the large variation in concentration of bile salts used for paediatric FaSSIF, led to two different bile salt concentrations as minimum and maximum value; 1.5 mM and 4.5 mM [Maharaj *et al*., 2016].

It is critical to further identify and quantify bile salts in the paediatric GI tract, as their role in oral drug absorption is acknowledged. Different patients' age groups may be characterised by different bile salts and bile salts level, therefore paediatric patients should be stratified according to age groups.

Work presented at

7. Chapter 7: Gastro-intestinal human samples analysis chapter includes characterisation of paediatric human intestinal samples in terms of pH, buffer capacity, osmolality. Bile salts have been also identified and quantified in the same samples by Dr. Pawar.

2.11 Impact of food on the GI environment in adults

Exploring and understanding the physicochemical properties of fasted and fed populations requires the combined work of several research groups, as reference meals vary. The composition of each meal, together with the timing that it was consumed by each population, are parameters that may affect greatly the physicochemical values, and subsequently, can have an impact on oral drug absorption.

There is ongoing discussion about the impact of food on the luminal environment. The importance of this matter is great as it may alter the strategy for new API development during preclinical studies. This is mainly because a fed or fasted environment can alter the intraluminal pH and thus, influence significantly the drug absorption. Furthermore, the impact of food on the luminal environment is significant for developing generic drugs [Pentafragka *et al*., 2018]. My paper [Papadatou Soulou *et al*., 2019] presented at 4, explores the gastrointestinal paediatric volumes at fasted and fluid fed state.

The table below (Table 2 1) is a summary of the main characteristics of gastric fluids that have been measured in adults at various times following administration of a glass of water and following administration of meal in the gastric and upper/ lower intestinal environment.

Table 2 1 Physicochemical properties and composition of the gastric contents in adults at over time after administration of i) a glass of water and ii) after administration of a meal to fasted adults. The composition, calorie content and calories origin is the same with the reference meal suggested by regulatory agencies (reference meal as described at the Drug Dissolution **and Bioavailability** section. Information taken from: Kalantzi *et al*., [2006]; Litou *et al*. [2016]; Petrakis *et al*. [2015]; Malagelda *et al.* [1979]; Koziolek *et al*. [2015]; Dressman *et al.,* [1990].

GASTRIC ENVIRONMENT	**Fasted State**		**Fed State**				
	10-20 min	**30-40 min**	**0.5 h**	**1 h**	**2 h**	**3 h**	**4 h**
pH	1.7-3.3	1.6-2.7	3.6-4.1	2.7-3.3	2-2.3	1.5-2.2	0.7-1.6
Buffer capacity (mmol/l/dpH)	4.7-21.3	18-27.6	25	23	23	29.8	-
Osmolality (mOsm/kg)	44.9- 103.6	117-178	531	474	474	321	-
Bile salts (mM)	0.014-0.032	0.013-0.147	<500 (LOQ) uM	<500 (LOQ) uM	<500 (LOQ) uM	<500 (LOQ) uM	<500 (LOQ) uM

Table 2 2 presents the physicochemical properties and composition of the upper intestinal contents at several timings in fasted and fed state.

Table 2 2 Physicochemical properties and composition of the upper intestinal contents about 30 minutes after administration of a glass of water and at various timings after meal consumption by fasted adults Dressman *et al*., [1990]; Hernell *et al*., [1990); Kalantzi *et al*., [2006]; Vertzoni *et al*., [2012]; Clarysse *et al*., [2009]; Riethorst *et al*., [2016]; Koziolek *et al*., [2015]; Litou *et al*., [2016]; Petrakis *et al*., [2015]; Moreno *et al*., [2006]; Bergstrom *et al*., [2014]; Fuchs *et al*., [2014].Duodenal contents have been hyperosmotic in most cases as shown below.

UPPER INTESTINAL ENVIRNOMENT (duodenum and proximal jejunum)	**Fasted State**	**Fed State**			
	30 min	**0.5 h**	**1 h**	**2 h**	**3 h**
pH	6.1-7	6.2-6.6	6.3-6.5	5.3-6.1	5.6-5.8
Buffer capacity (mmol/l/dpH)	6.9-9	28	22-27.4	18-23.3	12-25.6
Osmolality (mOsm/kg)	115-206	291-391	360-402	274-423	215-364
Bile salts (mM)	3.66-7.74	10.1-14	5-18.2	3.9-7.7	3.7-7.3

Table 2 3 Physicochemical properties and composition of the upper intestinal contents 5 hours after administration of a glass of water and 5 hours after administration of the reference meal to fasted adults [reference meal as defined by the regulatory authorities: two eggs fried in butter, two strips of bacon, two slices of toast with butter, four ounces of hash brown potatoes and a glass of whole milk] Diakidou *et al*., [2009]; Reppas *et al*., [2015]; Koziolek *et al*. [2015].

LOWER INTESTINAL ENVIRNOMENT (distal ileum and proximal colon)	**Fasted State**			**Fed State**		
	Distal Ileum	**Caecum**	**Ascending Colon**	**Distal Ileum**	**Caecum**	**Ascending Colon**
pH	7.7-8.1	7.4	7.8	8.1	6.4	6.0
Buffer capacity (mmol/l/dpH)	8.9	19.2	21.4	15.2	33.6	37.7
Osmolality (mOsm/kg)	60	144	81	252	267	224
Bile salts (mM)	71	183	115.2	182	280	587.4

The pH in the lower intestinal environment is higher than the upper intestinal environment for both fasted and fed states (Table 2 3). Interestingly, bile salts concentration is significantly elevated for the lower intestinal environment. Furthermore, the further lower the lower intestinal area, the highest the bile salt concentration (mM), ranging from 71- 587 mM.

2.12 Impact of malnutrition on paediatric oral drug absorption

Malnutrition is a serious condition that is common among the hospitalised, the elderly, pregnant and breastfeeding women and children [Neggers, 2016]. Children are more prone to malnutrition than adults, due to their increased nutritional needs [Blossner and de Onis, 2005]. Malnutrition includes the deficiencies, excesses and imbalances in the patient's intake of energy and/ or nutrients.

Until now, there is no review of the GI conditions in malnourished children. However, there is evidence that malnutrition can impact the GI environment, changing oral drug absorption [Freerks *et al*., 2019]. This can subsequently lead to alterations in the pharmacokinetics of the orally administered drug and finally, to a sub-therapeutic effect. My joint publication with the University of Greisfwald [Freerks *et al*., 2019] reviewed the impact of malnutrition on paediatric oral drug absorption. Reviewing paediatric malnutrition is crucial as the children who suffer from this disease are millions; at least 202 million children in world are stunted or wasted according to United Nations International Children's Emergency Fund (UNICEF)/ World Health Organisation [WHO]/World Bank Group Joint Child Malnutrition Estimates [dataunicef, 2018].

It has been shown that alterations in the paediatric oral cavity caused by malnutrition can lead to a reduced saliva secretion, which may subsequently lead to an impaired disintegration/ dissolution of the orally disintegrating dosage forms and swallowing. Additionally, changes in oral cavity attributed to malnutrition may cause decrease in saliva pH, buffer capacity and protein and enzyme levels. These changes may cause a limited impact on oral drug absorption [Ribeiro *et al.,* 2014; Bud *et al.,* 2017]. Finally, malnutrition may change the oral mucosa integrity, enhancing thus the potential for oro-mucosal drug absorption [Psoter *et al.,* 2008]. Malnutrition also changes the paediatric gastric environment, causing an increased gastric and reduced peptic activity, leading to limited dissolution of weakly basic drugs, that may limit the bioavailability, and to increased plasma levels of the

drugs, respectively [Gilman *et al.,* 1988; Gracey *et al.,* 1977]. Malnutrition impacts the paediatric small intestine as well. Decrease in the bile acids' levels are observed, leading to a reduced ability to solubilise poorly soluble drugs and further limit the bioavailability of the drugs. Reduced enzyme activity and an increased intestinal permeability has also been described [Thompson *et al.,* 1952; Behrens *et al.,* 1987; Lunn *et al*., 1991]. The decrease in the small intestinal surface area that has been reported leads to a reduced area for the overall absorption, which can ultimately lead to a decrease in the bioavailability of the drug [King *et al*., 2003; James *et al.,* 1972]. Chapter 3: Malnutrition chapter is a narrative review that links and summarises all the malnutrition data with regard to the paediatric GI tract and the oral drug absorption, that are available until now. The knowledge gaps are addressed so that future studies will focus on generating data to fill the missing parts and to build a complete profile of the GI alterations during the malnutrition state.

2.13 Existing Research Gaps on paediatric GI environment

The World Health Organization (WHO) highlight numerous deaths in children from low-income countries [Who.int., 2020], which are attributable to the impact of malnutrition on oral drug absorption and suboptimal dosing due to lack of paediatric gastrointestinal data. As a result, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) directed big pharma to focus on paediatric data generation for new and old medications [European paediatrics regulation in EU, 2007]. Thus, regulatory authorities asked big pharma and academia for generating data for paediatric GI volumes and unravelling the fluid distribution pattern in children's GI tract to explore if the fluid is distributed as fluid pockets across the paediatric GI tract. The information that will be generated, will be used by research groups in future to optimise the composition of simulated intestinal and gastric fluids and update the current PBPK models, which are crucial for oral drug absorption.

3 Chapter 3: Malnutrition chapter

"A review of gastrointestinal conditions critical to oral drug absorption in malnourished children"

3.1 Relevance to thesis

The focus of the thesis is oral drug absorption from the paediatric GI tract and how its anatomy and physiology affects oral drug absorption. In this chapter, we will explore the impact of malnutrition on oral drug absorption to provide us a deeper understanding of this very common condition in children and the affected organs in their body. Malnutrition massively affects children due to their high nutritional needs and is a huge health issue, which has global impact. According to the latest United Nations International Children's Emergency Fund (UNICEF)/ World Health Organisation (WHO)/World Bank Group Joint Child Malnutrition Estimates (2020), 6.9% of children worldwide are wasted, while 21.3% are stunted (The UNICEF/WHO/WB (JME), 2020). This makes the review of available data on the gastrointestinal anatomy and physiology of malnourished children relevant to the performance of orally administered medicines of high importance.

3.2 Aim of the study

The aim of this study was to review the available data on the gastrointestinal GI anatomy and physiology of malnourished children relevant to the performance of orally administered medicines. Specifically, the study aimed to explore the oral cavity, stomach, small intestine and large intestine of the malnourished children and the impact of malnutrition on these areas.

3.3 Objectives of the study

The objectives of this study were:

i) to identify relevant data via pubmed and scopus

ii) to conduct a narrative review to explore how the GI differences of malnourished children may affect oral drug absorption.

3.4 Study design

Statement: *"The author made a substantial contribution to the conception and design of the study, to the organisation of the conduct of the study, to carrying out the study and to analysis and interpretation of study data. Additionally, the author helped draft the output and critique the output for important intellectual content".*

The text below is taken from our joint publication (researcher Eleni Papadatou Soulou and supervisor Professor Hannah Batchelor) with the University of Greifswald (Lisa Freerks and supervisor Professor Sandra Klein). Authors Lisa Freerks and Eleni Papadatou Soulou worked together to generate the publication.

Eleni worked on the following sections by literature searching and data analysis: introduction, materials and methods, stomach, gastric contents, gastric emptying, small intestine, small intestinal contents, intestinal fluid output and dehydration in malnutrition, intestinal transit time, colon.

Lisa worked on the following sections: introduction, materials and methods, oral cavity, salivary secretion, saliva pH and buffer capacity, saliva composition, oral mucosa integrity, colon.

Professors Hannah Batchelor and Sandra Klein co-ordinated and supported the writing and edited the final version of the paper.

Reprinted with permission from Freerks L, Papadatou Soulou E, Batchelor H, Klein S. (2019). A review of GI conditions critical to oral drug absorption in malnourished children. Eur J Pharm Biopharm. (137), 9-22. doi: 10.1016/j.ejpb.2019.02.001. Epub 2019 Feb 5. PMID: 30735799.

Title: **A review of GI conditions critical to oral drug absorption in malnourished children.**

List of Authors: Freerks, L., Papadatou Soulou, E., Batchelor, H.K., Klein, S.

3.5 Abstract

Accurate prediction of oral absorption of drugs relies on bio-relevant methodology. Current methods are based on Western healthy adult populations. Malnourished children have many differences in their gastrointestinal anatomy and physiology compared to a healthy Western adult. These differences may affect the oral absorption of medicines and it is important to gather knowledge on these GI differences to develop bio-relevant predictive methods for this vulnerable population.

A literature search was conducted within PubMed and Scopus to identify papers that describe how gastrointestinal physiology and anatomy is altered in malnourished children. Relevant data was extracted, and a narrative review generated to describe how GI differences may affect oral drug absorption.

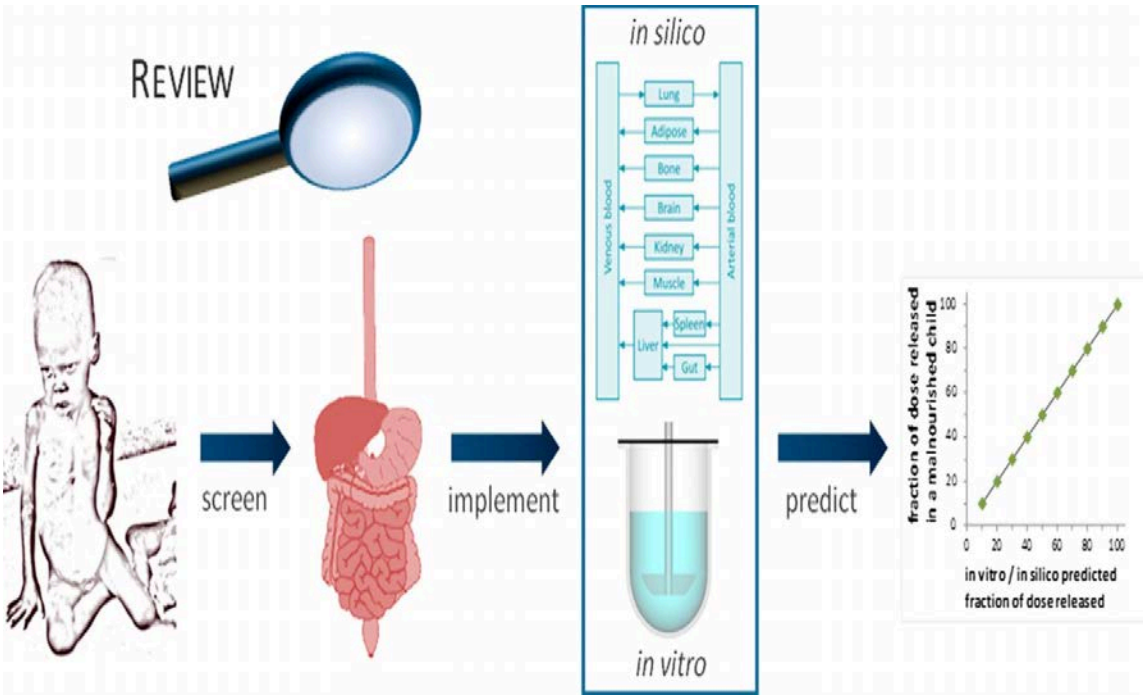
Several differences in GI anatomy and physiology were reported in the literature including: reduced saliva secretion; increased gastric pH; slower gastric emptying; increased levels of bacteria in the small intestine; reduced surface area of intestinal villi and increased intestinal permeability. Much of the data was more than 30 years old and referred to a heterogeneous malnourished population.

Sufficient data has been identified that will inform basic novel bio-relevant methods to predict oral drug absorption in malnourished children. Further work is required to generate additional data to improve these models and also to verify the models with appropriate pharmacokinetic data.

Keywords

Malnourished, biopharmaceutics, children, absorption, gastrointestinal physiology

3.6 Table of Contents Graphic



3.7 Introduction

When administering an oral drug product to a patient there are various factors which are important to ensure sufficient drug absorption; these include drug and formulation-related parameters like drug solubility and permeability, drug product dissolution and gastro- intestinal (GI) conditions including the composition and physicochemical properties of GI fluids, transit time and gut wall conditions relevant to metabolism, passive diffusion and active transport of drugs. GI physiology is therefore a key aspect that determines the *in vivo* performance of orally administered drugs.

Over the last decades, bio-predictive *in vitro* models have been established for predicting oral drug exposure for a number of compounds in adults after dosing either in fasted or fed conditions. There is a lack of such *in vitro* models for children, yet they are urgently required to better predict the *in vivo* performance of medicines in children to minimize the burden of clinical testing. When developing biorelevant *in vitro* media and models to simulate gastrointestinal conditions, the most appropriate environment needs to be represented. It is already known that there are

differences in the gastrointestinal physiology of children in different age groups, and in comparison to adults, which are accompanied by variable volumes, pH values and composition [Batchelor *et al*., 2014]; it is obvious that these differences will be different again in children suffering from diseases affecting GI conditions or in malnourished children.

Table 3 1 Most common used classification criteria of the nutritional status of children since 1956

<table>
<tr><th>Classification criteria</th><th>Definition</th><th>Type of classification</th><th>Advantages</th><th>Disadvantages</th></tr>
<tr><td>Gomez et al., 1956</td><td>body weight ranges as a percent of the theoretical average in relation to the age:
– protein energy malnutrition (PEM) I: 76 – 90 %
– protein energy malnutrition (PEM) II: 1 – 75 %
– protein energy malnutrition (PEM) III: ≤ 6 0 %</td><td>– quantitative classification into mild, moderate and severe cases
– indication for acute malnutrition (wasting)</td><td></td><td>– the percent-of-average method does not take into consideration the variability of the average
– in many communities the ages of children are not known
– application of the Gomez index requires the use of an external standard for comparison & different external standards are used in different studies</td></tr>
<tr><td>McLaren et al., 1967</td><td>scoring system (for details see the reference) for protein calorie malnutrition that includes: oedema; dermatosis; hair change and hepatomegaly in combination with measured serum albumin
– 0 - 3: marasmus
– 4 - 8: marasmic-kwashiorkor
– 9 - 15: kwashiorkor</td><td>– qualitative classification to distinguish marasmus, kwashiorkor and marasmic kwashiorkor</td><td></td><td></td></tr>
<tr><td>Wellcome classification, 1970</td><td>combines weight and the presence/absence of oedema to classify terminology
<table>
<tr><th rowspan="2">Weight* (% of standard)</th><th colspan="2">Oedema</th></tr>
<tr><th>Present</th><th>Absent</th></tr>
<tr><td>80 - 60</td><td>Kwashiorkor</td><td>Undernourished</td></tr>
<tr><td>< 60</td><td>Marasmic kwashiorkor</td><td>Marasmus</td></tr>
</table>
* standard = 50th percentile Boston values</td><td>– qualitative classification to distinguish marasmus, kwashiorkor and intermediate forms</td><td></td><td></td></tr>
<tr><td>Waterlow et al., 1976</td><td>according to Johansson et al., 1994 [19]: height-for-age
– Normal: > 95 %</td><td>– quantitative classification into mild,</td><td></td><td></td></tr>
</table>

	– Mild/PEM I: 90 - 95 % – Moderate/PEM II: 85 - < 90 % – Severe/PEM III: < 85 %	moderate and severe cases – indication for chronic PEM (stunting)		
National Center for Health Statistics (NCHS), 1978	categorization of the nutritional status of children with the aid of standards for – weight-for-age – length-for-age – weight-for-length – head circumference-for-age and Z (standard deviation)-scores: $\text{Z-score} = \frac{(\text{actual anthropometric value} - \text{median reference value})}{\text{standard deviation}}$ – 0.00 indicates a nutritional status equal to the median for a child of the same gender – ≤ 0.00 indicates a nutritional status that is the indicated number of standard deviations below the median for a child of the same gender and age: For example children of 0-5 years – severe malnutrition: any z-scores ≤ -2 – questionable malnutrition: any z-scores > -2 and ≤ -1 – normal weight: all z-scores > -1 – ≥ 0.00 indicates a weight-for-age that is above the median for a child	– quantitative classification into mild, moderate and severe categories	– Z scores reflect the reference distribution and are comparable across ages and indicators	– percent-of-median method does not take into consideration the variability in the relative width of the distributions of the different indicators – the data used to construct the reference came from a longitudinal study of children (0-3 years) from a single community in the United States. The children have rarely been measured (every 3 months), which is inadequate to describe the rapid and changing rate of growth in early infancy
WHO Child Growth Standards, 2006	categorization of the nutritional status of children with the aid of standards and Z-scores for: – length-for-age – height-for-age – weight-for-height – weight-for-age Z-score (WAZ) – weight-for-length Z-score (WLZ) – weight-for-height Z-score (WHZ)	– quantitative classification into mild, moderate and severe cases	– in contrast to NCHS standards these standards include children from around the world: Brazil, Ghana, India, Norway, Oman, USA – Z scores reflect the	

			reference distribution and are comparable across ages and indicators	
Body Mass Index (BMI)	weight in kg divided by square of height in m: – BMI percentile for age < 5: underweight – BMI percentile for age ≥ 5 or < 85: healthy weight – BMI percentile for age ≥ 85 or < 95: overweight – BMI percentile > 95: obese			

Malnutrition is a condition that is reported for several patients' groups and can be quite common in hospitalized and elderly patients [Hickson, 2006; Barker *et al.,* 2011; Agarwal *et al*., 2013], pregnant and breastfeeding women, and children [Neggers, 2016]. Due to their high nutritional requirements children are more susceptible to malnutrition than adults [Blossner and de Onis, 2005]. When designing appropriate bio-predictive *in vitro* models, it will be essential to consider the specific GI features of malnourished children. To date, a review of the GI conditions in malnourished children is not available. However, it is obvious, that malnutrition will alter the GI environment. An altered GI environment may affect pharmacokinetics of orally administered drugs, which in turn may affect efficacy and subsequent therapy.

In basic terms, the absorbable dose is influenced by the transit time; concentration of drug at the absorbing surface; surface area available for absorption and the permeability of the drug across the membrane. Differences in any of these parameters will affect the fraction of dose absorbed; hence predictions made in a healthy population may be very different to those where the GI parameters are different. A detailed understanding of the nature of specific GI parameters in malnourished children and of how much they may affect timing, site and extent of *in vivo* drug release seems to be one of the key factors for developing better oral medications for these children. Moreover, any *in vitro* and/or *in silico* models that consider these relevant GI parameters and would be applicable to predict the *in vivo* performance of orally administered drugs in malnourished children would be extremely beneficial with regard to increasing the safety and efficacy of oral

medicines provided to this patient group.

The group of malnourished patients describes a heterogenous population. Malnutrition refers to deficiencies, excesses or imbalances in a person's intake of energy and/or nutrients. Malnutrition is divided into undernutrition, which includes stunting, wasting and underweight, and to overweight, which includes obesity and several diet relevant diseases as heart disease and cancer. Undernutrition is caused by a lack of food in general, but also by inadequate, unhealthy diets and diseases including digestive and absorptive disorders resulting in micronutrient deficiencies. It makes children in particular much more vulnerable to disease and death and is responsible for approximately 50% of child mortality for those under five years old [dataunicef, 2018]. According to the latest United Nations International Children's Emergency Fund (UNICEF)/ World Health Organisation (WHO)/World Bank Group Joint Child Malnutrition Estimates (May 2018) at least 202 million children worldwide are stunted or wasted [dataunicef, 2018].

In the literature, different criteria that have been applied to classify the nutritional status of children can be found (Table 3 1). According to current WHO criteria undernutrition can be present in 4 broad sub- forms, i.e. underweight (low weight-for-age), stunting (low height-for-age), wasting (low weight-for-height) and deficiencies in vitamins and minerals [WHO: malnutrition, 2017]. The main hallmark of child malnutrition is growth retardation. Underweight, which represents low weight for age [WHO: malnutrition, 2017], is defined as a weight-for-age at least two standard deviations below the median weight based on the WHO child growth standards [Gordon *et al*., 2012; WHO, 2018]. "Stunting" is a term for chronic undernutrition and reflects low height for age [WHO: malnutrition, 2017]; defined as less than two standard deviations (SD) below the WHO standards [Gordon *et al*., 2012; WHO, 2018]. Acute undernutrition is termed "wasting", where moderate wasting is indicated by a weight between two and three SDs below the WHO standards [Gordon *et al*., 2012; WHO, 2018]. Severely wasted children have a weight of less than three SDs below the WHO standards or a mid-upper arm circumference (MUAC) of less than 115 mm in children of 6–60 months

[Gordon *et al*., 2012; WHO: child growth standards, 2017; WHO, 2018].

"Severe wasting" and bilateral oedema are independent diagnostic criteria for severe acute malnutrition (SAM), which is associated with a high risk of death and which requires urgent therapeutic feeding [WHO: child growth standards, 2017]. Protein-calorie malnutrition (PCM) is a specific subtype of SAM with a deficiency in macronutrients including protein, carbohydrates and fat. The WHO defines PCM, also known as protein energy mal- nutrition (PEM), as "a pathological condition that results from a lower ingestion of protein and calories, which occurs more frequently in children under five years of age". Severe PCM can be categorized into three principal clinical forms: (i) marasmus, an acute malnutrition characterized by severe wasting of fat and muscle and a gross under- weight status; (ii) kwashiorkor presents with moderate growth retardation and bilateral pitting oedema; and (iii) marasmic kwashiorkor, the most severe form of PCM, a mixed form of both marasmus and kwashiorkor that is characterized by the presence of both wasting and bilateral pitting oedema [Rodriguez *et al*., 2011]. PCM is a major public health problem affecting a high proportion of infants and older children worldwide and accounts for a high childhood morbidity and mortality in the developing countries. Its association with a wide spectrum of infections necessitates multiple drug therapies. Whereas the epidemiology of PCM has been extensively studied globally, the pathophysiological changes that may affect disposition of these drugs in malnourished children have not been reviewed in that much detail [Oshikoya and Senbajo, 2009]. Oshikoya *et al*. [2010] performed a systematic literature re- view to determine the effects of PCM on drug pharmacokinetics and concluded that there have been relatively few pharmacokinetic studies of drugs frequently used for treatment of children with PCM. However, they also present case examples in which decreased absorption of drugs such as carbamazepine [Bano *et al*., 1986], chloroquine [Walker *et al.,* 1987], sulphadiazine [Menta et al., 1980], and chloramphenicol [Eriksson *et al.,* 1983] have been reported in children with PCM when compared with healthy normal children and was attributed to morphological changes in the jejunum. In addition, treatment failure with artemether-lumefantrine was reported to be due to incomplete absorption in a malnourished

child [Valecha *et al.*, 2009].

The aim of this review was to review the available data on the gastrointestinal anatomy and physiology of malnourished children relevant to the performance of orally administered medicines. The focus was set on the screening of the particular features of undernutrition. Thus, in the following sections, the terms malnutrition and undernutrition can be regarded as interchangeable.

3.8 Materials and methods

A literature search was conducted within PubMed and Scopus using the search terms listed in Figure 3 1 . Literature that provided original data on parameters that affect GI physiology associated with oral administration and absorption of drugs were included in further analysis. Specifically, literature that reported aspects on gastrointestinal transport, motility or contents was sought. Studies where children had co-morbidities were also included in the analysis.

The search was conducted between September 2017 and March 2018. The search results were sorted by the authors (Lisa Freerks and Eleni Papadatou Soulou) based on titles, abstracts or full text articles. Additional literature was obtained from reference lists of the identified articles or reviews on this topic.

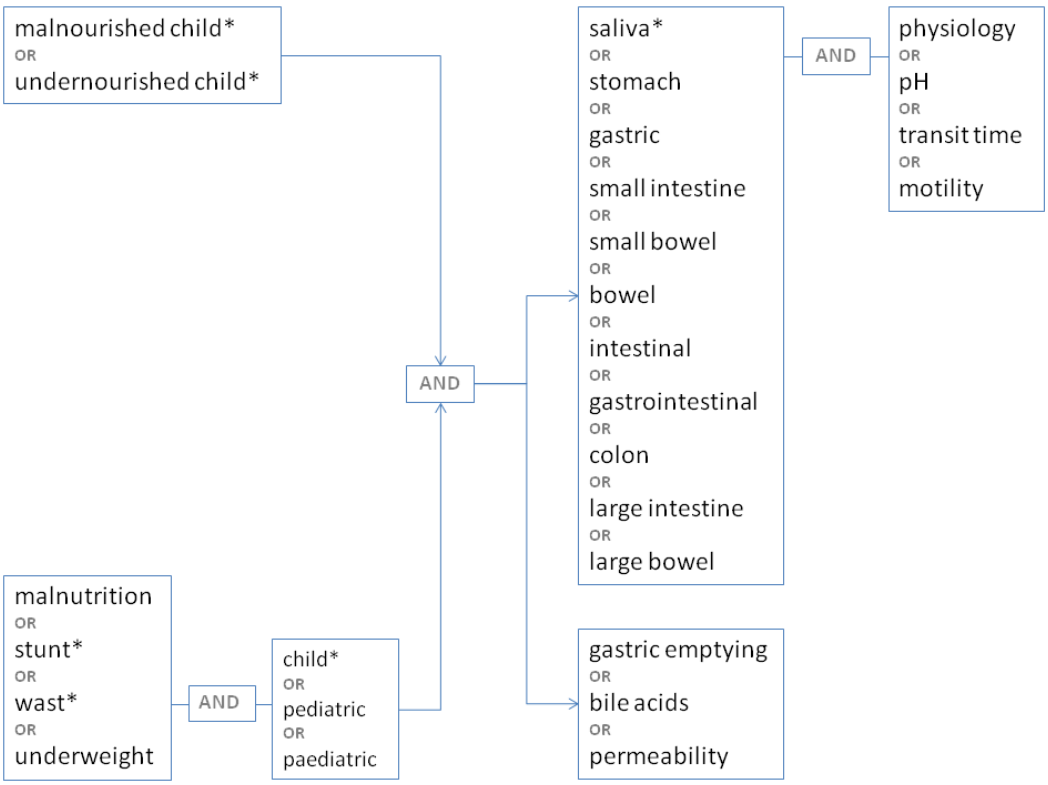


Figure 3 1 Search terms used within the literature search

For data extraction, studies were sorted according to whether they investigated oral cavity, gastric, small intestinal or large intestinal parameters. Further sorting permitted papers to be reviewed based on physiological parameters of these regions. Data was extracted from relevant papers to gather evidence that was summarized for each absorption parameter. There was too much heterogeneity in the identified literature which did not permit a systematic meta-analysis. Therefore, the results could not be presented as a systematic review. Thus, a narrative review was selected to discuss reported effects of malnutrition that would impact on oral drug absorption considering the process from the initiation of oral ingestion to excretion.

3.9 Results

Definition of malnutrition used in the papers identified.

The wide range of criteria used to classify malnutrition was highlighted in Table 3 1 and the papers identified used a range of these criteria when reporting results. The

NCHS criteria were the most commonly used with the Gomez index, the Wellcome classification and WHO child growth standards being used in multiple studies [Johansson, 1994; Boaz *et al*., 2013; Hossain *et al*., 2010; Hossain *et al*., 2016l Wessels *et al*., 2013; Agarwel *et al*., 1984; McMurray *et al*., 1977; Johnsson *et al*., 1992; Franco *et al*., 1985; Franco *et al*., 1986; Psoter *et al*., 2008; Goto *et al.,* 2002; Myo Khin *et al.,* 1999; Gilman *et al.*, 1988; Sullivan *et al*., 1992; Behrens *et al*., 1987; Shaaban *et al*., 2004; Gracey *et al*., 1977; Mehta *et al*., 1984]

3.9.1 Oral cavity

The oral cavity is the site of administration for oral medicines. Solid oral dosage forms are usually designed to be swallowed whole and to release the drug in the stomach and/or intestine. However, in children solid oral dosage forms might not be swallowed as rapidly as in adults. Thus, there is a chance that conditions in the oral cavity such as pH, buffer capacity and composition of saliva affect both integrity and drug release from solid oral dosage forms and consequently affect *in vivo* drug release. Dosage form integrity could be affected by mechanical forces caused by tongue and palate as well as by the volume, pH, buffer capacity and composition of saliva. Salivary pH and buffer capacity also play an important role in dental diseases and compared to healthy children a higher incidence of caries and other dental diseases have been observed in children with poverty driven malnutrition [Ribeiro *et al*., 2014; Bud *et al*., 2017].

Saliva parameters

Table 3 2 Alterations in a) salivary secretion, b) pH and buffer capacity, and c) salivary composition

a) Summary of data on changes in salivary secretion

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Agarwal *et al*., 1984	52 patients and 42 controls, age: 1-10 years	Gomez index (weight-for-age): mild, moderate and severe malnourished	time of saliva collection	longer collection time of defined volume of saliva in severe malnourished children	small study, correlation and regression analysis

Johansson *et al.*, 1992	34 patients and 34 controls, age: 8-12 years	Gomez index (weight-for-age): mild, moderate and severe malnourished	saliva collection	reduced stimulated saliva secretion	small study, well described study, statistical analysis
Psoter *et al.*, 2008	1017 Haitian children age: 11-19 years	NCHS (weight-for-age): severe and questionable malnourished	saliva collection	reduced saliva secretion	retrospective cohort design, loss of follow-up not comprehensible, large patient group, methods well described, statistical analysis

b) Summary of data on changes in salivary pH and buffer capacity

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Bud *et al.*, 2017	37 patients and 87 controls, age: 6-12 years	Body Mass Index: underweight	CRT buffer strips	reduced saliva pH, reduced saliva buffer capacity (not statistically significant)	small study, statistical analysis
Johansson *et al.*, 1992 and 1994	34 patients and 34 controls, age: 8-12 years	Gomez index (weight-for-age): mild, moderate and severe malnourished	final pH after air was bubbled for 20 min into a mixture of saliva and HCl	salivary buffer capacity decreased with increasing level (severity) of malnutrition, however, subjects in the control group had a markedly lower buffer capacity which was most probably due to genetic/ethnic effects (African study group vs. Swedish control group)	small study, well described study, statistical analysis

c) Summary of data on changes in saliva composition

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Agarwal *et al.*, 1984	52 patients and 42 controls, age: 1-10 years	Gomez index (weight-for-age): mild, moderate and severe malnourished	Protein measurement with Folin-phenol reagent (Lowry *et al.*, 1951), Measurement of enzyme arginase activity with centrifuge column technique (Gopalakriskna and Nagarajan, 1979)	reduced saliva protein and enzyme levels	small study, correlation and regression analysis

Johansson *et al.*, 1994	34 patients and 34 controls, age: 8-12 years	Gomez index (weight-for-age): mild, moderate and severe malnourished	Coomassie blue method, degradation of insoluble blue-colored starch polymer, anatomic absorption, and other	no effect on saliva protein and enzyme levels, reduced concentrations of calcium and chloride	small study, well described study, statistical analysis
McMurray *et al.*, 1977	44 patients and 27 controls, age: 1,5-2 years	Gomez index (weight-for-age): mild and severe malnourished	IgA concentrations with Immunodiffusion plates, Protein measurement with Folin-phenol reagent (Lowry *et al.*, 1951), aminopeptidase measurement by hydrolysis of L-methionyl β-naphthylamide in Tris-HCl buffer at pH 8.0	reduced saliva IgA concentrations, no effect on saliva protein and enzyme levels	small study, statistical analysis, Classification system of malnourished children: Gomez index → no standard defined

Increased residence time and drug release in the oral cavity can increase systemic drug absorption across the oral mucosa. Therefore, mucosal integrity is also an important fact to consider when discussing drug administration to malnourished children.

3.8.1.1 Salivary secretion

Salivary secretion has been reported to be lower in malnourished compared to healthy children (Table 3 2). Agarwal *et al*. reported that saliva collection took longer (30–40 min) following stimulation with citric acid and strawberry flavour in malnourished children compared to control children or children (aged 1–10 years) with milder forms of PEM (10 min) [Agarwal *et al*., 1984]. Other research found impaired saliva flow rates in severely malnourished children (aged 8–12 years and 11–19 years) in both unstimulated and stimulated conditions [Johansson *et al*., 1992; Psoter *et al*., 2008]. Saliva flow rates were reported to be linked with level of malnutrition and the reduction in flow rate was related to the severity of PEM [Psoter *et al*., 2008] However, it should be noted, that malnutrition is often linked with dehydration which in- dependently of malnutrition is known to affect saliva secretion

rate [Fischer and Ship., 1997; Walsh *et al*., 2004].

3.8.1.2 Saliva pH and buffer capacity

The pH of saliva was reported to be lower (6.9 ± 1.85) in underweight children compared to children of a normal weight (8.1 ± 1.95) (aged 6–12 years) [Bud *et al*., 2017] (Table 3 2). In addition, a reduction in salivary buffer capacity has been reported for this patient group [Johansson *et al.,* 1992; Johnasson *et al.,* 1994; Bud et al., 2017].

3.8.1.3 Saliva concentration

Concentrations of sodium, potassium, phosphate, hexosamines, fucose, sialic acid and total protein as well as amylase activity in mal- nourished children have been reported to be similar to those of a control group (aged 8–12 years) [Johansson *et al*., 1994] (Table 3 2). However, in the mal- nourished group (aged 8–12 years) significantly lower concentrations of calcium and chloride as well as a decreased amount of protein secreted per minute were found in paraffin-stimulated whole saliva [Johansson *et al*., 1994]. Agarwal *et al*. detected a continuous fall of protein, ferritin and arginase activity in stimulated saliva that correlated with the severity of PEM [Agarwal *et al*., 1984]. Analyses of unstimulated whole saliva show contradictory results regarding the concentrations of total IgA: while Johansson *et al.* detected no differences in the concentrations of total IgA between the PEM group and the control group; McMurray *et al.* found reduced IgA concentrations in malnourished children (aged 1.5–2 years) [Johansson *et al*., 1994; McMurray *et al*., 1997]. Salivary total protein, albumin, and aminopeptidase were found in similar concentrations in all children by McMurray *et al*. and, Johansson *et al*. detected lower concentrations of anti-*S. Mutans* IgA and lactoferrin as well as lower activity of bacteria agglutinating protein (BAGP) in the PEM group.

3.8.1.4 Oral mucosa integrity

PEM appears to have multiple effects on the oral tissues and oral disease development [Psoter *et al*., 2008]. Besides deficiencies in protein malnourished children PEM results in a lack of a number of essential vitamins and minerals that

effect structures in the oral cavity. These include folate and other B complex vitamins; vitamins A, C, and D, calcium and fluoride. Consequently, besides impaired dentition, the nutritional status of the body is also associated with disturbances in other oral structures and presents with recurrent apthous stomatitis, atrophic glossitis, painful, burning tongue characterized by inflammation and defoliation of the tongue, mucosal atrophy and oral ulcers [Psoter *et al*., 2008; Ehizele *et al*., 2009; Sheetal *et al*., 2013]. There is very little data reported on the extent to which oral mucosa integrity is impaired in malnourished children. However, there is evidence that long-term chronic malnutrition causes a significant reduction in resistance and progressive damage to the oral mucosa that will result in reduced resistance to colonization and invasion of pathogenic microorganisms [Slotwinska *et al*., 2014] and consequently will most probably also enhance permeability and therefore oromucosal drug absorption.

In summary, the imbalance between the supply of the nutrients and the body's demand results in several oral manifestations that could alter oral drug absorption. However, whereas the impacts on dentition are usually irreversible, in children rehabilitation will most likely result in complete recovery of oral mucosa integrity, saliva secretion and composition.

3.8.2 Stomach

Volume, composition and physicochemical properties of gastric fluids are important factors regarding drug solubility and/or disintegration properties of solid oral dosage forms and thus can affect *in vivo* drug release. The motility pattern in the stomach and the secretion of gastric juices are directly dependent on food intake. Thus, it is necessary to differentiate between the fasted and fed state. Different nutrition habits or long periods of fasting lead to the assumption that gastric conditions in malnourished children may differ from conditions in healthy children, which may result in altered drug performance.

3.8.2.1 Gastric Contents

Several studies have shown correlations between reduced fasted gastric acid secretion and malnutrition (Table 3 3). Gracey *et al*. detected, that unstimulated

gastric acid secretion was reduced in 9 of 14 malnourished children, while maximal acid output (60 min after pentagastrin stimulation) was below normal in all malnourished patients (aged 7–54 months) [Gracey *et al*., 1977]. Gilman *et al*. reported that the fasted gastric acid output was lower in severely malnourished Bangladeshi children compared to better nourished children (0.22 vs 0.52 mEq HCl/h) (aged 3.3 ± 2.4 years) [Gilman *et al.,* 1988]. In the children enrolled in the Gilman study, three weeks of nutritional rehabilitation lead to equivalent rates of fasted gastric acid secretion in the two groups showing that this is a reversible phenomenon [Gilman *et al.,* 1988]. At this point, it also should be noted that as for saliva secretion, impaired gastric secretion may relate to both malnutrition or dehydration of the children, which often cannot be clearly distinguished. Thus, the increase in gastric acid secretion could be also an effect of rehydration. Shashidhar *et al*. detected a decreased mean acid concentration in malnourished children (aged 12–60 months), here divided into kwashiorkor and marasmic children, under unstimulated and stimulated fasted conditions, although results show a high inter-individual variability [Shashidhar *et al*., 1976].

Under unstimulated fasted conditions the pH of gastric juice was above 4 in 76% of malnourished children; After nutritional rehabilitation this decreased to 69% in malnourished children compared to 55% in better-nourished children (aged 3.3 ± 2.4 years) [Gilman *et al*.,1988]. Under stimulated conditions, the percentage of pH values above 4 was reduced: 26% in malnourished children, 24% in children after nutritional therapy and 0% in better-nourished children [Gilman *et al*.,1988]. However, pH of gastric juice under stimulated fasted conditions is not equal to gastric pH under fed conditions. Thus, also the composition and quantity of food has an important impact on gastric pH, especially when considering that there are various types of diets worldwide.

Peptic activity was reported to be significantly reduced in malnourished children under fasted conditions compared to well-nourished children (aged 12–60 months) [Shashidhar *et al*., 1976] (Table 3 3). Yet, shortly after stimulation of gastric secretion peptic activity rose in all children al- though it was still significantly decreased in malnourished children.

The reduced gastric output observed at baseline in severely mal- nourished children correlates to a higher incidence of infections [Martinsen *et al*., 2005] credited to the loss of the gastric acid barrier. The relationship between *H. pylori* infection and acidity within the stomach is complex; a low level of acidity in the stomach may increase the risk of infection in the gastrointestinal tract and also overgrowth of intestinal bacteria that may lead to diarrhea. The prevalence of *H. pylori* in children is high in developing countries and since *H. pylori* infection is associated with an increased gastric pH, it might be associated with malnutrition [Gilman *et al*., 1988; Sullivan *et al*., 1990; Sharmen *et al*., 2002].

Gastric parameters

Table 3 3 Alterations of a) gastric contents, b) gastric emptying

a) Summary of data on changes in gastric contents

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Gracey *et al.*, 1977	14 patients and 21 controls, age: 7-54 months	Wellcome classification: marasmus and kwashiorkor	volumes were measured and the HCl content was estimated by titration to pH 7.4	basal gastric acid output was below normal in 4 of the 7 infants, in all patients maximal acid output was reduced	small study, no publication of measured values in control group
Gilman *et al.*, 1988	35 patients and 20 controls, age: 3,3 ± 2,4 years	NCHS (weight-for-height): marasmus, marasmic kwashiorkor and kwashiorkor	volumes were measured and the HCl content was estimated by titration to pH 7.0	increased gastric pH, basal volume of gastric secretion was significantly lower in malnourished children, stimulated acid concentration and volume of gastric secretion was significantly diminished in malnourished children	small study, well described study, follow-up, statistical analysis
Shashidhar *et al.*, 1976	30 patients and 12 controls, age: 12-60 months	Recommendation of Indian Academy of Pediatrics (1972): marasmus, kwashiorkor	pH meter and titrating with NaOH, colorimetric method with haemoglobin	reduced acid secretion/increased gastric pH, reduced peptic activity	small study, inclusion criteria of patients not comprehensible (recommendations of Indian Academy of Pediatrics), lack of information regarding the statistical test

b) Summary of data on changes in gastric emptying

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Shaaban *et al.*, 2004	27 patients and 15 controls, age: 11,97 ± 6,03 months	Wellcome classification: marasmus, marasmic kwashiorkor and kwashiorkor	ultrasonographic examination	delayed gastric emptying of marasmus and marasmic kwashiorkor patients, not statistically significant delay of gastric emptying of kwashiorkor patients, significant increase after nutritional rehabilitation	small study, well described study, follow-up, statistical analysis
Franco *et al.*, 1985	9 patients and 7 controls, age: 5-29 months	Gomez index (weight-for-age): severe malnourished and McLaren (1967): kwashiorkor, marasmic kwashiorkor and marasmus	double sampling technique with gastric tube (George *et al*., 1968)	volumes left in the stomach of children with marasmus upon admission to the hospital were significantly lower than those observed in the controls, no difference between children after nutritional rehabilitation and controls	small study, quality of method questionable, patients treated with different helminthics such as thiabendazole, which can evoke unspecific gastrointestinal disorders, follow-up, statistical analysis
Franco *et al.*, 1986	22 patients and 7 controls, age: 7-45 months	Gomez index (weight-for-age): severe malnourished and McLaren: kwashiorkor, marasmic kwashiorkor and marasmus	double sampling technique with gastric tube (George *et al*., 1968)	no differences of gastric emptying between kwashiorkor patients and control group, not statistically significant delay of gastric emptying of marasmic kwashiorkor patients	small study, quality of method questionable, patients treated with different helminthics such as thiabendazole, which can evoke unspecific gastrointestinal disorders, follow-up, statistical analysis
Brunser *et al.*, 1990	-	-	-	slow gastric emptying	review no reference indicated

In conclusion, available literature reports reduced gastric acid secretion in malnourished children, yet nutritional rehabilitation seems to result in an immediate improvement. It should be noted that nutritional rehabilitation typically results in rehydration of malnourished children. Malnourished children with watery diarrhea are usually treated with Rehydration Solution for Malnutrition (ReSoMal) [WHO: malnutrition, 2016]. Hence, the increase in gastric acid secretion

can be caused by both nutritional re- habilitation and rehydration.

3.8.2.2. Gastric emptying

Investigations on gastric emptying in malnourished children show contradictory results (Table 3 3). Brunser *et al*. within a review paper stated without indicating a reference, that there are clinical observations showing that severe cases of malnutrition are characterized by gastric dilatation, slow emptying and vomiting [Brusnet *et al*., 1990]. Two studies investigating gastric emptying in marasmic, kwashiorkor and marasmic kwashiorkor children (aged 5–29 months and 7–45 months) showed that children with marasmus showed faster gastric emptying of a 5% (w/v) glucose solution administered at a volume of 20 mL/kg body weight compared to a control group. Severely malnourished children with kwashiorkor had no detectable abnormalities in gastric emptying, while in marasmic kwashiorkor children a tendency of delayed gastric emptying was observed [Franco *et al*., 1985; Franco *et al*., 1986]. However, the power of these studies is limited, as the patient group was treated with different helminthics, such as thiabendazole, which can evoke unspecific gastrointestinal disorders. In contrast, a significantly delayed gastric emptying in both marasmic and marasmic kwashiorkor children (aged 11.97 ± 6.03 months) was reported by Shaaban *et al* [2004]. In an ultrasonographic study they investigated the impact of nutritional re- habilitation on gastric emptying using a liquid (infant powdered milk formula) and a semisolid meal (milk, rice and high protein additive). They found a clear delay in gastric emptying of both liquid and semi- solid food in marasmic and marasmic kwashiorkor children when compared to a control group (aged 11.97 ± 6.03 months). As could be expected, the delay in the gastric half emptying time was higher with the semisolid than with the liquid meals [Shaaban *et al*., 2004]. Nutritional rehabilitation was observed to speed up gastric emptying after 30 ± 7 days of nutritional treatment [Shaaban *et al*., 2004].

3.8.3 Small intestine

The small intestine is the site where maximal drug absorption occurs. Therefore, small intestinal conditions, such as luminal contents, surface area, transit time and

mucosal permeability, can affect drug absorption and this information can help to develop predictive *in vitro* test designs as accurately as possible.

3.8.3.1 Small intestinal contents

The small intestinal contents influence the solubility of drugs which in turn will influence their absorption. Key components of small intestinal fluids are bile, enzymes and bacteria. Mehta *et al*. analyzed the duodenal contents following a milk stimulus from sixty marasmic children between the ages of 9–42 months. Results showed significantly lower mean concentrations of conjugated bile acids (1.36 mg/mL vs. 2.92 mg/mL) and total bile acids in marasmic children compared to well-nourished children, while the concentration of free bile acids was increased (0.60 mg/mL vs. 0.06 mg/ mL) (Table 3 4). Unfortunately, measured pH values weren't published, but the low pH of duodenal juice was mentioned [Mehta *et al*., 1984]. Duodenal aspirates were analyzed from 18 severely protein calorie malnourished children (aged 15–64 months) to measure the contents with respect to the ability to achieve micellar solubilisation of lipids. The results showed that micellar lipid and fat absorption were low in PCM children due to lower levels of bile salts [Scheider *et al*., 1974]. Children under 5 years of age with severe acute malnutrition were shown to have higher total serum bile acids compared to controls which relates to lower biliary secretion within the GI tract [Zhang *et al*., 2016]

Sauniere *et al*. analysed the duodenal contents of 25 children (1 month to 8 years) with acute symptoms of kwashiorkor. Bicarbonate and volume of duodenal juice of malnourished children were not different compared to the normal African population, while pancreatic enzymes, such as amylase and lipase, were significantly decreased, except for trypsin, which was not affected [Sauniere *et al*., 1986]. These results agree with the findings from 40 children (aged 9–51 months) with kwashiorkor where a wide variation in significant depression of enzyme activity was observed [Thompson *et al*., 1952] (Table 3 4).

Underproduction of acid in the stomach is the most likely cause of the bacterial overgrowth observed in the small intestine of under- nourished children. The impact of high levels of bacteria in the small intestine include impaired nutrient absorption

and risks of sub-efficacious oral vaccination in children aged 2 months to 5 years [La gos *et al*., 1999]. Several studies have reported higher than usual levels of viable bacteria within the small intestine in malnourished children (aged 9 months to 6 x observed [Thompson et al., 1952]Table 3 4

3.8.3.2 Intestinal fluid output and dehydration in malnutrition

The adult small intestine receives large quantities of fluid via dietary fluid intake or as secretions from salivary glands, stomach, pancreas, liver and the small intestine itself. The small intestinal epithelium absorbs about 6–7 L of fluid per day. Only about 1.5 L enters the large intestine and are further reduced to less than 250 mL/day that are excreted with feces. If an individual has diarrhea then osmoregulation is affected and the fluid within the intestine is not reabsorbed and the total intestinal fluid output is elevated to balance the osmolality to favour the absorption [King *et al*., 2003]. The small intestine is typically a site of net water absorption which has been observed in children with malnutrition. However, in cases of malnutrition that is associated with diarrhea a net secretion of water was measured within the jejunum [James *et al*., 1972]. This alteration from net absorption to net secretion is likely to reduce the uptake of drugs within the GI tract in cases of diarrhea.

3.8.3.3 Intestinal permeability

Several studies done in the 1960–1970s showed that the shape of the small intestinal wall changed from regular long villi in healthy patients (villi are structures within the small intestine that increase the small intestinal surface area and, consequently, absorption) to irregular broader and shorter villi in malnourished children (0–3 years) and that the shape in malnutrition is associated with an overall smaller surface area compared to healthy tissue [Mata *et al*., 1972] (Table 3 4). However, increased permeation of cellular materials was seen suggesting that the intestine is more permeable in malnutrition [Mata *et al.,* 1972; Durban, 1965; Campos *et al*., 1979]. As the severity of malnutrition increases so do the changes in the intestinal epithelium as the abnormalities in marasmus were mild in comparison to children (aged 0–3 years) with kwashiorkor [Campos *et al*., 1979]. Atrophy of villi in malnutrition has also been reported [Campbel *et al*., 2003; Farras *et al*., 2018; Durban, 1965]

The Lactulose/Mannitol intestinal permeability test (L:M) is a commonly used technique to measure small intestinal function and can relate to changes in: (a) small intestinal epithelial area; (b) transcellular and paracellular transport and (c) damage and permeability. Mannitol can be used as a marker to assess the mucosal absorptive area and lactulose to assess the integrity of the intestinal membrane. In some studies, a lactulose/rhamnose test is used; rhamnose is similar to mannitol in that it is a monosaccharide and mucosal damage will reduce mannitol/rhamnose absorption whilst increasing permeability of lactulose.

A typical reference value for L:M is 0.09 as this was defined as an upper normal limit in 30 healthy Dutch children (aged 0–16 years) [Van Elburg *et al*., 1995]; values greater than 0.09 suggest enteropathy is present. Typically, lactulose (which is large) relates to the overall integrity of the membrane and mannitol can relate to the surface area or overall absorptive capacity of the membrane.

Small Intestinal parameters

Table 3 4 Alterations in a) bile concentrations, b) enzyme concentrations, c) bacteria, d) intestinal epithelium, e) intestinal permeability

a) Bile concentrations

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Mehta *et al*., 1984 [40]	60 patients and 15 controls (normal age-matched), age: 9-42 months Study conducted in Rohtak, India	Gomez index <60 % of weight for age standard	Analysis of duodenal aspirates for free and conjugated bile acids by thin-layer chromatography	Mean concentration of conjugated bile acids as well as total bile acids was significantly lower in marasmic children compared to normal age-matched children The concentration of free bile acids, was significantly higher in marasmic children compared to normal age-matched children	Small study Statistical analysis
Schneider and Viteri, 1974 [48]	18 patients with severe PCM aged 15-64 months and 4 controls	Weight for height (76 % for PCM children)	Analysis of duodenal aspirates for bile acids	Lower levels of conjugated bile acids (3.07 in PCM +diarrhea; 8.28 in PCM – diarrhea	Small sample population Statistical analysis

	(healthy children aged 17-23 months)			compared to 10.96 μM/mL in controls) yet higher levels of free bile acids (1.68 in PCM +diarrhea; 0.78 in PCM – diarrhea compared to 0.62 μM/mL in controls)	
Redmond *et al.*, 1972 [46]	20 patients with kwashiorkor and 10 controls (children with gastro-enteritis); age: 3-24 months Study conducted in the South African Medical Research Council Clinical Nutrition Research Unit at the University of Cape Town and the Red Cross War Memorial Children's Hospital	Kwashiorkor stated but definition not stated	Analysis of duodenal aspirates for bile acids	Free bile acids were detected in 8/20 patients with Kwashiorkor and 2/10 with gastro-enteritis (the control group)	Very small study No statistical analysis

L:M test: Lactulose/ Mannitol intestinal permeability test , L:R test: Lactulose/ Rhamnose intestinal permeability test

b) Enzyme concentrations

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Sauniere *et al.,* 1986 [43]	11 patients (from the Ivory Coast) with Kwashiorkor and 23 controls (French children); age: 1month to 8 years	Not stated	Analysis of duodenal aspirates	A decrease in lipase, amylase, chymotrypsin and phospholipase was found in children with Kwashiorkor compared to the control group	Small sample population Statistical analysis
Thompson and Trowell, 1952 [44]	40 patients aged 12-51 months and 24 controls (in patients without diagnostic criteria for kwashiorkor); age: 9-51 months Study conducted at Mulago Hospital and the	"Established kwashiorkor"	Analysis of duodenal aspirates	A reduction in the concentration of amylase and lipase in children with Kwashiorkor compared to the control group	Small sample population Statistical analysis

	Department of Medicine, Makerere College, Kampala, Uganda				

c) Bacteria

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Omoike and Abiodun, 1989 [50]	Nigerian children aged 2 months – 5 years Well-nourished diarrhea-free Nigerian children were controls compared to (i) well-nourished children with acute diarrhea and (ii) malnourished children with or without diarrhea 50 children in total	Well-nourished vs kwashorkor; marasmus; marasmic kwashiorkor +/- diarrhoea	Analysis of duodenal aspirate	Higher levels of bacteria were found in the small intestine of malnourished children Well-nourished children had bacterial counts $<10^5$ organisms /mL In malnourished children counts were 10^3-10^9 organisms/mL	Small sample population Statistical analysis
Heyworth and Brown, 1975 [47]	Gambian children aged 9-34 months (n=25) Those with chronic diarrhea were compared to those with acute diarrhea	Marasmus; Kwashiorkor; marasmic kwashiorkor	Analysis of duodenal aspirate	Significantly higher levels of bacteria ($>10^5$ organisms/mL) were found in the small intestine of 22/25 malnourished children; chronic diarrhea is associated with increased levels of bacteria	Small sample population Statistical analysis
Mata *et al*., 1972 [51]	13 patients with acute PCM and 4 controls (normal); age: 1-6 years Study conducted in Clinical Center of the Institute of Nutrition of Central America and Panama	PCM characterized by a marked growth retardation	Analysis of gastric, duodenal and jejunal aspirates	Those with malnutrition **plus diarrhea** showed higher levels of bacteria in their stomach and jejunum yet there was no difference for those with malnutrition (without diarrhea) compared to controls	Small sample population No statistical analysis

d) Shape of intestinal epithelium

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Campbell *et al*., 2003 [52]	38 Gambian patients and 19 age-matched	Weight z score, height z score and BMI z score	Biopsy taken and microscopic analysis of	Villous/crypt ratio was 0.80 in Grade 1 PEM; 0.84 in grade 2 PEM. Gambian	Small sample population Statistical analysis

	UK controls; Age: 0-3 years		villous height and crypt depth	controls had villous/crypt ratio of 0.81 yet in UK controls the ratio was 2.1.	
Farràs *et al*., 2018 [53]	15 Zambian patients with persistent diarrhea; mean age 15 months	WHO Child Growth Standards	Biopsy taken and microscopic analysis of villous height and crypt depth	Clear evidence of villous blunting and shorter villus height compared to literature controls	Small study, no controls No statistical analysis
Burman *et al*., 1965 [60]	17 children <3 years from Nairobi with kwashiorkor and children from England as controls	Kwashiorkor	Jejunal biopsies	The jejunal tissue showed more ridges and fewer fingers in children with Kwashiorkor compared to controls	Small study with biopsies

e) Changes in intestinal permeability

Reference	Population	Malnutrition definition used	Methodology	Finding	Quality of Study
Hossain *et al*., 2016 [54]	925 children aged 13.2 ±5.2 months from a Bangladeshi slum	WHO (Weight for age; height for age; weight for height; mid-upper arm circumference	Lactulose/Mannitol intestinal permeability test (L:M)	44 % had enteropathy (leaky membrane) as reflected by a L:M of ≥0.09 Younger age and having diarrhea increased the risk factors for having enteropathy	Robust methodology; large population; well described study
Brewster *et al*., 1997 [55]	149 children aged 26.7-29.9 months with Kwashiorkor and 45 inpatient controls in Malawi	Kwashiorkor	Lactulose/Rhamnose intestinal permeability test (L:R) L:R test	The initial geometric mean L-R ratios (×100) (with 95% confidence interval) in kwashiorkor were 17.3 (15.0 to 19.8) compared with 7.0 (5.6 to 8.7) for controls	Robust methodology; large population; well described study
Goto *et al*., 1999 [56]	158 Guatemalan infants <12 months from low-income, periurban community of Guatemala City	Length for age; weight for age; weight for length	Lactulose/Mannitol intestinal permeability test (L:M)	30 % had leaky intestinal permeability (L:M≥0.07) The L:M in currently asymptomatic infants who had diarrhea during the week before testing (0.087; CI = 0.49, 0.154) was higher (0.087) than that in children who had been free from diarrhea for at least 1 week (0.052).	Robust methodology; large population; well described study

				Younger age and having diarrhea increased the risk factors for having enteropathy	
Behrens *et al*., 1987 [57]	68 Gambian infants aged 0–18 months	Marasmus with weight for age at <60 %	Lactulose/Mannitol intestinal permeability test (L:M)	Those with marasmus had significantly higher L:M compared to others L:M ratio was 1.3 in malnourished population compared to 0.42 in well-nourished children and 1.0 in children with chronic or acute diarrhea	Good statistical analysis and follow-up
Lunn *et al*., 1991 [58]	119 Children from 2-10 months in Gambia	Height and weight	Lactulose/Mannitol intestinal permeability test (L:M)	The L:M ratio could predict 43 % of the observed variation in length and 39 % of the observed variation in weight growth This shows that growth is related to intestinal permeability where enteropathy limits growth	Statistically strong study
Campbell *et al*., 2003 [52]	73 children aged 8-48 weeks from rural Gambia	Height and weight	Lactulose/Mannitol intestinal permeability test (L:M)	Higher permeability reported to correlate to the severity of malnutrition	Robust study
Boaz *et al*., 2013 [59]	26 south Indian children aged 6-59 months hospitalized for management of acute gastroenteritis plus 20 controls	Malnutrition was defined as a weight for age Z score below -2SD by WHO	Lactulose/Mannitol intestinal permeability test (L:M)	61.5% of children with malnutrition and acute diarrhea and 32.2% of children without diarrhea had increased intestinal permeability.	Robust study
Hossain *et al*., 2010 [61]	77 children 13.1 ±4 months severely malnourished Bangladeshi children and 17 aged match controls	weight-for-age Z-score (WAZ) < -3 in relation to the WHO 2006 standard	Lactulose/Mannitol intestinal permeability test (L:M)	Eighty-four percent of the children had L/M ≥ 0.07, suggestive of impaired intestinal function The L:M ratio of malnourished children was greater (0.09) compared to controls	Robust study

Manary *et al.,* 2010 [62]	25 asymptomatic Malawian children aged 3-5 years risk for tropical enteropathy and zinc deficiency	Weight and height	Lactulose/Mannitol intestinal permeability test (L:M)	88% of children had abnormal L:M ratio (>0.10) L:M was directly correlated with endogenous faecal zinc and negatively correlated with net zinc retention	Robust study
Johansen *et al*., 1989 [63]	Children aged 1 month – 3 years (well-nourished (n=17) and severely malnourished (n=9)	<<60 % standard weight for age	Absorption of Polyethylene glycols from 292-1250 Da, Measured by determination in urine	Reduction in permeability of PEGs was observed in severely malnourished children Diarrhea exacerbated this effect	Good methodology

A study on 97 severely underweight Bangladeshi children (mean age 9 years) showed that the difference in mannitol absorption was greater than in lactulose absorption in malnourished children compared to healthy children; this relates to the loss of surface area which is consistent with the shape changes in the villi [Hossan *et al*., 2010]. Intestinal biopsy studies [Durban, 1965; Stanfield *et al.,* 1965] in the villi [Hossan *et al.,* 2010]. Intestinal biopsy studies [Durban, 1965; Stanfield *et al.*, 1965] in malnourished children show that kwashiorkor is associated with villous atrophy, decreased villous-crypt ratio and increased cellularity of the lamina propia; these changes impact the absorptive capacity as well as the overall surface area. Several studies have been conducted to investigate the intestinal permeability of malnourished children with or without diarrhoea as well as before and after nutritional rehabilitation [Boaz *et al*., 2013; Hossain *et al.,* 2010; Hossain *et al*., 2016; Sullivan *et al*., 1992; Behrens *et al*., 1987; Brewster *et al.,* 1977; Brewster *et al.,* 1997]. The damaged villi lead to a compromised intestinal wall that can be associated with absorption of large macromolecules that lead to immune- and inflammatory diseases. These diseases further damage the epithelial wall and reduce the barrier for absorption of drugs. Chronic intermittent diarrhoea may be linked to carbohydrate malabsorption within the intestine due to the rapid intestinal transit times associated with diarrhoea that may limit overall absorption in addition to the differences in the permeability across the membrane [Campos *et al.,* 1979]. Brewster *et al*. showed that the combination of increased lactulose permeation and decreased

L-rhamnose absorption results in a higher prevalence of diarrhoea [Brewster *et al.,* 1997]. Behrens *et al*. found repeatedly increased intestinal permeability to disaccharides in a number of tests on 68 Gambian infants (aged 0–18 months), most of whom had at least one episode of diarrhoea [Behrens *et al.,* 1987]. These findings lead to the conclusion that diarrhoea is associated with mucosal damage, which results in compromised small intestinal barrier function and uptake of larger molecules, such as lactulose, through the site of damage [Lunn *et al*., 1991]. Therefore, diarrhoea seems to correlate with abnormally high L/R – ratios and thus with an increased intestinal permeability. Indeed, L/R – ratios of patients with diarrhoea were observed to be above normal in several studies and correlated with the duration and frequency of diarrheic events and returned to normal with resolution of diarrhoea [Boaz *et al*., 2013; Behrens *et al*., 1987; Brewster *et al*., 1997]. It can be stated that malnutrition itself is associated with changes in the intestinal mucosa resulting in a reduced absorptive area and malabsorption, while diarrhoea, which is often linked to malnutrition, is associated with mucosal damage. Estimates of the prevalence of impaired intestinal permeability (higher values of L:M) range from 62 to 96% that results in poor growth among infants and young children in developing countries in sub- Saharan Africa and Asia (urinary L:M concentration ratio > 0.10–0.12) [Goto *et al*., 2002; Manery *et al*., 2010; Lunn, 2000]. Perturbed intestinal function is associated with the malabsorption of macro- and micronutrients, including fat, carbo- hydrates, and vitamins A, B12 and folate [Baker, 1976; Brown *et al*., 1979; Chacko *et al*., 1984]. Wessells *et al*. [2013] study looked at zinc absorption in Burkinabe children (6–23 months) and also measured the lactulose/mannitol measures to assess intestinal integrity. The results showed that the absorption of zinc was linked to the L:M permeability test with a greater proportion of normal children showing higher levels of zinc absorption, however this was only significant different for the most severely affected children [Wessels *et al*., 2013].

3.8.3.4 Intestinal transit time

The transit time through the small intestine is an important factor regarding the absorption of nutrients and drug products, as it affects the time that food and drugs

have contact with the absorptive epithelium. In 1999 there was one study conducted regarding orocecal transit time in malnourished children. Myo-Khin *et al*. [1999] used the lactulose breath hydrogen test to investigate orocecal transit in 90 Myanmar children between the ages of 1 and 5 years. After classification, according to weight-for-age, length-for-age and weight-for-length indices, 31 children were defined to be malnourished, whose orocecal transit time was compared to transit time in well-nourished children. No significant difference between malnourished and well-nourished children was found. However, children with a history of diarrhea were excluded from the study. As diarrhea and vomiting are two of the most common problems of PEM, as subsequent symptoms, a reduced retention of drugs and decrease of the transit time through the bowel can be expected [Oshikoya and Senbajo, 2009].

3.8.3 Colon

No literature was identified that reported colonic transit time and colonic permeability in malnourished subjects. There are some studies suggesting that malnutrition is a risk factor for an increased incidence or duration of diarrhoea [Sepulveda *et al*., 1988; Palmer *et al*., 1976; Fuchs and Victora, 2002; Guerrant *et al*., 1992]. As a consequence of diarrhoea, colonic transit time in malnourished children might be shorter than in healthy children. However, some of the currently available reports on this topic are contradictive and the information available to date is not sufficient for a final conclusion. Another important point to consider in this discussion would be the alterations in the gut microbiome that are reported for severely malnourished children [Subramanian *et al*., 2014]. Future research should thus focus on expanding the knowledge in this field.

3.10 Discussion

Malnutrition can affect the gastrointestinal tract and subsequently, modify drug absorption. Therefore, malnutrition can have an indirect impact on drug toxicity or suboptimal therapeutic effects. Clearly, there is a pressing need to understand the impact of malnutrition in drug absorption. Table 3 4 summarizes the findings from the literature and provides some implications for drug absorption in malnourished

children.

Table 3 5 Potential impacts of specific features on oral drug absorption in malnourished children

Gastro-intestinal region	Impact in malnourished children	Consequences for oral drug absorption
Oral cavity	Reduced saliva secretion	Impaired disintegration/dissolution of orally disintegrating dosage forms and swallowing
	Reduced saliva pH	Limited impact on oral drug absorption
	Reduced saliva buffer capacity	
	Reduced saliva protein and enzyme levels	
	Impaired oral mucosa integrity	Increased chance for oromucosal drug absorption
Stomach	Increased gastric pH/ Reduced acid secretion	Limited dissolution of (weakly) basic drugs may limit bioavailability
	Reduced peptic activity	Increased plasma levels of drugs that are prone to enzymatic degradation
	Unknown impact on gastric emptying (yet compromised in vomiting/diarrhoea)	Risk of an increased variability in plasma profiles
Small intestine	Reduced bile acid concentrations	Reduced ability to solubilise poorly soluble drugs and may reduce overall bioavailability
	Reduced enzyme activity	Limited impact on drug absorption except in cases where prodrugs that require enzymatic cleavage are used
	Increased permeability	Enhanced absorption of certain drugs due to increased permeability; increased bioavailability may lead to toxicity or the need for dose titration
	Reduced surface area	Reduced area for overall absorption may reduce overall bioavailability, particularly in cases of diarrhea
Colon	No evidence found	Unknown

The data identified within this search was generally quite old. Some general trends towards alterations in oral, gastric and small intestinal physiology were identified. Next to no information on the colon, another vital section of the GI tract, was available. There is very limited novel data available to really understand the implications of mal- nutrition on the absorption of orally administered medicines, yet this can be of great importance for such a vulnerable population. A systematic review on the impact of malnutrition on the pharmacokinetics of drugs used in children reported that the extent of absorption was increased in 8 drugs, yet this was attributed to be likely due to changes in clearance rather than the GI physiological changes [Oshikoja, 2010]. However, these statements should be handled with care, since these 8 drugs had significantly different properties with

regard to molecular weight, aqueous solubility and permeability in adults and represented candidates from all classes of the biopharmaceutics classification system (BCS). Consequently, at least for some of these APIs plasma levels in malnourished children are likely to be affected by GI physiology. Moreover, for several other compounds, examples where absorption in malnourished children was unchanged or reduced can be found in the literature [Bano *et al.*, 1086; Walker *et al.*, 1987; Menta *et al.*, 1980; Eriksson *et al.*, 1983].

Current *in vitro* tools that are used to predict oral drug absorption are based on adult physiologies and Western populations. The simulated gastrointestinal media and biorelevant dissolution methods available to date address GI conditions in fasted and fed healthy European subjects [Vertzoni *et al.*, 2005; Vertzoni *et al.,* 2010; Dressman *et al.*, 1998; Jantratid *et al.*, 2008; Klein, 2010]. Biorelevant *in vitro* tools addressing the particular GI features in malnourished children are currently unavailable. Therefore, there is a real need to develop biorelevant *in vitro* methods that reflect the conditions found in malnourished children and different types of malnutrition to better understand how drugs are absorbed in these critical and vulnerable populations. This will ensure the selection of adequate doses ensuring a safe and effective drug treatment of these children. Biorelevant *in silico* tools using physiologically based pharmacokinetic modelling can provide a tool to better understand the disposition of drugs within a range of populations. There has been some work looking at simulating a malnourished population to explore drug pharmacokinetics [Thuo *et al.*, 2011]. *In silico* tools permit exploration of predicted pharmacokinetics when considering each different aspect of GI physiology/anatomy for the population under investigation which can provide details on sensitivity to change based on the type of drug presented. Further work is required in this area.

3.11 Conclusions

Despite the significance of malnutrition as a global health issue this population has been neglected when considering efficacy of medicines used to treat malnourished children. Oral drug treatment during the acute phase of malnutrition needs to be

carefully considered to ensure that the therapy is efficacious. Further work is required to develop biorelevant models that incorporate the findings from this review and to verify these *in vitro* and *in silico* models using appropriate pharmacokinetic data. All methods need to acknowledge the heterogeneity of malnutrition as a disease state as well as the age-related changes associated with paediatric populations.

3.12 Acknowledgements

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3.14 Conflict of interest

The authors declare no conflicts of interest

4 Chapter 4: Magnetic Resonance Images (MRI) analysis chapter

'Magnetic resonance imaging quantification of gastrointestinal liquid volumes and distribution in the gastro-intestinal tract of children

4.1 Relevance to thesis

The focus of the thesis is the healthy paediatric GI tract and how its anatomy and physiology affects oral drug absorption. This chapter provides information on the volume and location of fluid in the paediatric gastro-intestinal tract, exploring this way and generating data on the complex paediatric GI tract. Little is known so far for the fluid volume and its localisation in the paediatric GI tract, as most studies have explored the adult scenario. It is proven that in adults, fluid exists in "pockets" across the small intestine, thus this has to be validated or rejected for children population as well. Knowledge extracted from this chapter aims to inform the design of *in vitro* and *in silico* models that predict the absorption of oral drugs administered to children.

4.2 Synopsis

Table 4 1Study Summary of the MRI analysis study

Study Title	Measurement of fluid volumes and localisation within the gastrointestinal tract of children using MRI data.	
Internal ref. no. / short title	RG 17-057	
Study Design	Observational Study	
Study Participants	Children who require MRI as part of their clinical care and the gastrointestinal tract is visible. The children will be stratified using the WHO criteria: Neonate: 0-30 days Infant: 1 month-2 years Young child: 2-6 years Child: 6-12 years Adolescent: 12-18 years	
Planned Sample Size	Similar studies use 10-12 participants for characterisation. We plan to obtain a similar sample size for each age stratification. We will not end recruitment at 10-12 as we do not yet know what the variability will be within the population therefore this is a minimum target sample size.	
Planned Study Period	30 months starting in January 2018	
	Objectives	**Outcome Measures**
Primary	To measure the volume of fluid within the gastrointestinal tract (stomach and small intestine) of children	Measurements of the mean and range of fluid volumes within the

		stomach and small intestine of children
Secondary	To measure the localisation of fluid within the gastrointestinal tract of children	Measurements of the mean and range of fluid pockets distributed within the GI tract of children

4.3 Aim of the study

The aim of this study was to provide information about the freely available fluid, in the form of fluid pockets, in the paediatric GI tract (specifically stomach and small intestine) with regard to volume and its location.

4.4 Objectives of the study

The primary objective of the study was to measure the mean and range of fluid volumes within the stomach and small intestine of children.

The secondary objective was to quantify the mean and range of number of fluid pockets distributed within the GI tract. Furthermore, the location of the fluid pockets (duodenum, jejunum, and ileum) was recorded. Data from fed and fasted children were compared and the observed differences were explored.

4.7 Study design

This study used MRI data that is captured as part of routine clinical care for children. Therefore, this study is an observational, prospective study with non-probability sampling. The project was ethically approved REC reference: 18/EM/0251 (IRAS 237159 MRI: Fluid volumes and localisation in paediatric GI tract).

This work has been published and the following text is directly taken from the publication.

Further details on the methodology used to identify the fluid pockets and to record the fluid volumes is provided following the paper (4.16.1.1 MRI Images Analysis Protocol and 4.16.1.2 ImageJ analysis protocol)

Title: 'Magnetic resonance imaging quantification of gastrointestinal liquid volumes and distribution in the gastro-intestinal tract of children"

List of authors: Papadatou-Soulou, E., Mason, J., Parsons, C., Oates, A., Thyagarajan, M. and Batchelor, HK.

Statement: *"The author made a substantial contribution to the conception and design of the study, to the organisation of the conduct of the study, to carrying out the study and to analysis and interpretation of study data. Additionally, the author helped draft the output and critique the output for important intellectual content".*

4.6 Abstract

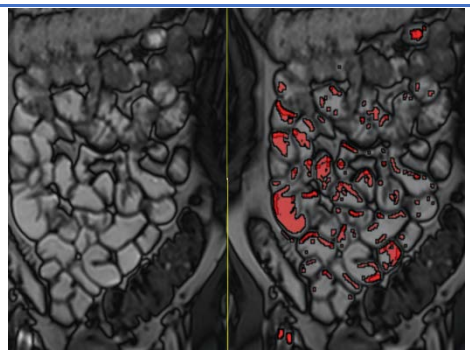
The volume and localisation of fluid in the paediatric gastro-intestinal tract is crucial to inform the design of *in vitro* and *in silico* models that predict the absorption of oral drugs administered to children. Previous studies have used magnetic resonance imaging (MRI) to quantify fluid volumes and localisation in the intestines of adults; this study is the first to undertake similar analysis of paediatric participants. This study quantified the amount and distribution of fluid in fasted and fluid-fed children using MRI data captured during routine clinical assessment.

Data from 32 fasted children (aged 0-16 years) and 23 fluid-fed (aged 8-16 years) was evaluated. The gastric volume ranged from 0-9mL in the fasted and 19-423mL in the fluid-fed state. The small intestinal volume was recorded to be 0-51mL in the fasted and 6-91mL in the fluid-fed state with an average number of 7.7 and 22.4 fluid pockets respectively. The data showed significant differences in gastric volumes and the number of fluid pockets in the small intestine for age-matched fasted and fluid-fed children ($p<0.05$). Both the number and the volume of pockets reported in children are much lower than those previously reported in adults.

This study is the first to report intestinal volumes and localisation in children and provides new information to inform the design of bio-relevant *in vitro* models and real values to update *in silico* models. The availability of data from both fluid-fed and fasted children show the extremes of fluid volumes that are present in the gastro-intestinal tract which is useful to understand the variability associated with drug absorption in children.

Keywords: gastric fluid; intestinal fluid; MRI; children; bio-relevant dissolution

4.7 Table of Contents Graphic



MRI to image fluid pockets in children

4.8 Introduction

Oral drugs are often administered as solid dosage forms (e.g. suspensions; tablets or capsules) where disintegration and dissolution are essential prior to absorption. Variable absorption profiles are often observed that are attributed to individual inconsistencies within the gastro-intestinal environment [Kimura and Higaki, 2002]. Significant efforts have been made to develop *in vitro* and *in silico* models that replicate the GI environment to better understand the processes of disintegration and dissolution with a view to minimising the variability associated with absorption. Typically, pharmacokinetic profiles in children are predicted based on extrapolation from adult data based on body mass or surface area algorithms rather than using physiological data. However, for oral absorption knowledge of the GI fluid is important to predict performance in children.

Prior to absorption of orally administered drugs, medicines often have to undergo disintegration and dissolution within the gastro-intestinal tract. The impact of the volume and composition of gastro-intestinal (GI) fluids will influence the processes of disintegration and dissolution and can affect the subsequent absorption profile. The volume of fluid used within *in vitro* testing systems varies from low values (<50mL) in bio-relevant testing systems to 900mL for pharmacopoeial methods as limited data exist on relevant values to use. The biopharmaceutics classification system uses fluid volume as a key parameter to classify drugs where the dose number (D_0) describes the volume in which the maximum dose strength is soluble; this is used to classify medicines as either highly or poorly soluble. The D_0 used in the calculations for determining BCS criteria is determined by the formula:

Equation 4 1

$$D_0 = \left(\frac{M}{V}\right) \Big/ C$$

where M is the dose strength of the oral dosage form (mg), V is the volume administered (mL), and C is the drug's solubility (mg/mL).

A dose number value greater than 1 indicates a poorly soluble drug whereas less than 1 is highly soluble. A highly soluble drug is likely to be well absorbed as solubility within the GI environment is not a rate limiting step for absorption. In the USA and in Europe a volume of 250mL (representing a glass of water) is used to calculate the dose number, whereas in Japan the volume is lower at 150mL to account for reduced volumes of water co-administered in clinical testing protocols [Kuribayashi *et al.,* 2016]. Extrapolation of adult dose numbers to

paediatric populations has been undertaken to obtain age-appropriate fluid volumes administered using body surface area [del Moral Sanchez *et al*., 2018; Gandhi e*t al*., 2014] giving values of 33.6mL for a neonate; 66mL for an infant and 127.6mL for a 7-year old child. Other literature reported a "worst-case" scenario of 25 mL as an administration volume for all paediatric subgroups [Abden Rahman *et al*., 2012]. Normalisation of the residual gastric fluid volume using extrapolation from adults on a mL per Kg basis gave gastric fluid volume values of 14.9mL in neonates; 29.8mL in a 6-month old infant and 86.8mL in a 7 year old child [Shawahna *et al.,* 2016]. A recent study on mini-pigs showed that gastric fluid volume was linearly related to the weight of the pig which suggests that weight is a suitable measure to use to extrapolate gastric fluid volumes [Guo *et al.,* 2018].

Knowledge of the volume and composition of fluids in the gastro-intestinal environment are critical to prediction of absorption as they provide an understanding of the media for disintegration and dissolution which can be replicated in *in vitro* and *in silico* models. Significant efforts have been made to measure both volume [Grimm *et al*., 2018] and composition of gastro-intestinal fluids in adults [Reppas *et al.,* 2015; Riethorst *et al*., 2016; Clarysee *et al.,* 2009; Fuchs *et al.,* 2014] and to a more limited extent children [Van Den Abeele *et al.,* 2018]. Fluid within the adult small intestine has been reported to be present in pockets rather than homogenously distributed along the GI tract. This distribution pattern will further complicate the absorption profile and adds to variability observed in pharmacokinetic profiles from orally absorbed medicines [Schiller *et al*., 2005]. The comparative gastric and small intestinal volume values in children are likely to have a substantial impact on the rate and extent of drug absorption following oral administration which provides a more physiological approach to extrapolation of data.

The impact of gastro-intestinal GI fluid volume on predictions of pharmacokinetic profiles has been shown to be significant for poorly soluble drugs in adult populations [Sutton *et al*., 2009]. Therefore, it is critical that representative fluid volumes are used to predict absorption for all populations. The range of fluid volumes from the fasted to fed state is also of interest as the variation can impact upon the absorption of medicines. Therefore, knowledge of the fluid volumes in the extremes of fasted and fed states is of interest.

Previous studies conducted in adults demonstrated the value of magnetic resonance imaging (MRI) to accurately measure the volume and localisation of fluid within the gastro-intestinal tract [Grimm *et al*., 2018a; Grimm *et al.,* 2018b; Mudie *et al.,* 2014; Schiller *et al.,* 2005]. Many

children undergo MRI as part of their clinical care where their abdomen is visible. This study sought ethical approval to use data from clinical MRI procedures in children to measure fluid volumes and localisation in the stomach and small intestine of these paediatric patients. The primary outcome measure was to quantify the mean and range of fluid volumes within the stomach and small intestine of children. Secondary outcome measures were to quantify the mean (and range) number of fluid pockets distributed within the GI tract of children and to record their location (duodenum, jejunum and ileum). The study also explored the difference in these outcomes from fluid-fed and fasted participants.

4.9 Materials and Methods

This study was an observational, retrospective study with non- probability sampling. The project sites were two major teaching Hospitals in the West Midlands (University Hospitals Coventry and Warwickshire NHS Trust and Birmingham Children's Hospital). The project was ethically approved REC reference: 18/EM/0251 (IRAS 237159 MRI: Fluid volumes and localisation in paediatric GI tract).

4.9.1 Study Participants

Anonymised MRI data sets were provided to the researchers from children (from 0-16 years) who required MRI for clinical purposes where the abdomen (stomach and small intestine) were clearly visible. The two sites involved had different protocols for the MRI procedure: one required children to ingest 500mL Oral Klean Prep (a macrogol solution) in the 60 minutes prior to their MRI (hereon referred to as fluid-fed children); the other site required children to fast overnight prior to the MRI.

In order to ensure that the patient population was as close to "normal-healthy" as possible, the following exclusion criteria were used, patients with: acute abdomen (appendicitis or perforated viscous); malignant bowel disease; surgery (bowel section, excluding appendicectomy); bowels wall thickening/ stricture/ fistula/ abscess.

4.9.2 Magnetic resonance Imaging (MRI)

MRI scanning was performed at two sites using different apparatus, the methods of subsequent analysis accounted for these differences in equipment used to acquire images. This imaging process provided clear images where fluids can be clearly distinguished.

The fluid fed participants were scanned at University Hospital Coventry and Warwickshire using either a 1.5T MR imaging unit (Optima MR450w, GE Healthcare, Chicago, USA) with a 48-channel body coil where the MRI protocol used was a coronal balanced steady-state gradient echo sequence (FIESTA) (slice thickness= 4.0 mm; echo train length = 1; intersection gap = 5.0mm; matrix size, 512 x 512; field of view, a x b cm; TR/TE, 5.7/1.9 ms). Or the scan was conducted using a 1.5T MR imaging unit (Aera, Siemens Healthcare, Erlingen, Germany) using a body coil and the MRI protocol used was a coronal balanced steady-state gradient echo sequence (True FISP) (slice thickness = 6.0 mm; echo train length = 1; intersection gap = 3.0mm; matrix size, 256 x 256; field of view, a x b cm; TR/TE, 652.8/2.1 ms).

The fasted participants were scanned at Birmingham Children's Hospital using either a 1.5T MR imaging unit (Siemens MAGNETOM Avanto 1.5T MRI System, USA) with a 16-element parallel imaging receiver coil where the MRI protocol used was a T2 SPACE coronal sequence (no gap between slices; slice thickness= 0.90 mm; matrix size = 0.8 x 0.8mm; field of view = 250; TR/TE = 1700/98 ms). Alternatively, a 1.5T MR imaging unit (Aera, Siemens Healthcare, Erlingen, Germany) using a 16-element parallel imaging receiver coil was used where the MRI protocol used was a coronal T2 SPACE sequence (no gap between slices; slice thickness, 0.9 mm; matrix size = 0.8 x 0.8 mm; field of view = 400; TR/TE = 2000/241 ms).

T2 SPACE is a variant of a three dimensional (3D) turbo spin echo sequence. Compared to a conventional turbo spin echo sequence, T2 SPACE uses non-selective, short refocusing pulse trains that consist of radiofrequency pulses with variable flip angles. This allows for very high turbo factors (> 100) and high sampling efficiency. A SPACE sequence produces high resolution isotropic images which can be reconstructed in multiple planes. SPACE sequences are less sensitive to susceptibility, flow and chemical shift artefacts which make it superior over the conventional turbo spin echo. By using a SPACE sequence, it was possible to produce isotropic 0.9mm 3D T2 contrast images in less than 7 minutes.

4.9.3 Image analysis

Horos software was used to visualise the MRI date sets. Horos is a free and open source code software (FOSS) program that is distributed free of charge under the LGPL license at Horosproject.org and sponsored by Nimble Co LLC d/b/a Purview in Annapolis, MD USA. The fluid within images was identified using the cerebrospinal fluid as a reference point for each data set; this approach acts as an internal control for each data set and represents the "free"

water present [Luoma et al., 1997; Hoad et al., 2007]. This value was used to provide a threshold to enable the fluid in the MRI data set to be clearly visualised. In brief, the image slice that most clearly showed the CSF within the spinal canal was selected to generate the threshold value. A rectangle was drawn entirely within the CF and HOROS software was used to calculate the signal intensity of this area to set the limit for free fluid within the MRI data set. In this study, signals from the stomach and the small intestine were manually identified whereas the kidneys, gallbladder, bladder and visible vessels were manually excluded from subsequent analysis. Representative images are shown Figure 4 1. The gastro-intestinal fluid was identified in each slice manually and the area of each fluid pocket was calculated following transfer of the image into ImageJ [Abramoff *et al*., 2004]. The volume of each pocket was determined by multiplication of the slice thickness by the area measured as previously described [Grimm *et al*., 2018b]. Using each patient's individual data, it was possible to create 3D MRI images (using Horos software) that assisted in mapping the GI anatomy which was particularly useful in the determination of the location of the identified fluid pockets. The 3D images were used to identify fluid pockets that overlapped into adjacent slices to better calculate the volume of each continuous pocket. For each participant, the total number of pockets was recorded as well as the volume and location of each pocket. Location of pockets within the small intestine was based on the visual distinction of small and large bowel, based on anatomical knowledge and observed differences after thresholding the slices using HOROS software. It was challenging to identify where the duodenum, jejunum and ileum start and finish, as there is no specific small bowel length for all subjects. Approximation of the location of fluid pockets was undertaken by dividing the total small bowel into two parts; the upper left-hand corner in an X, Y coronal plane was used as the starting point and moving in a diagonal line the upper left 2/5 were designated to be the duodenum and jejunum; the lower right hand 3/5 of the image represented the ileum; this was used to then determine where the fluid pockets were located.

All analysis was undertaken by a single operator initially with a sub-sample of 10% of all participants being checked by a second operator to ensure that there was consistency (±10%) in the measurements obtained.

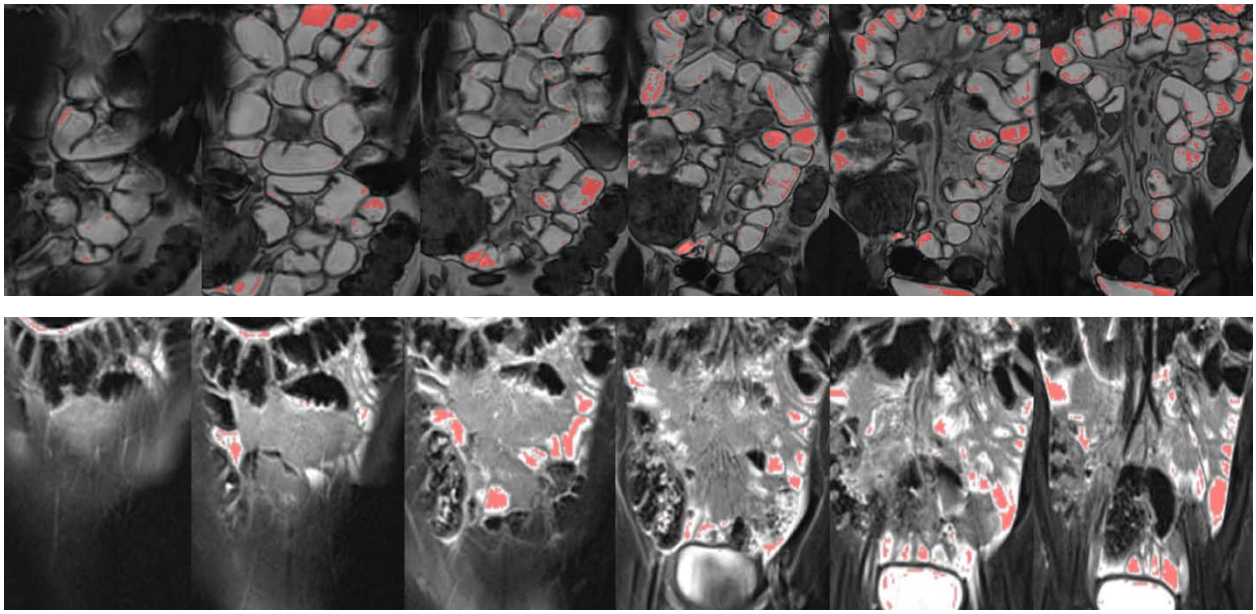


Figure 4 1 Example MRI data from two participants; the images show a sequence of slices in the coronal plane moving from the front to the back (spine) of the subject; the red areas are the fluid areas thresholded and included in further analysis. The upper image set is from a fluid-fed 16-year old and the lower imaged from a fasted 8 year old.

4.9.4 Statistical analysis

The individual volumes and number of fluid pockets were plotted to provide data on individual variability. The data are also shown as the median, interquartile values and full range.

Statistical tests were performed using SPSS. An independent samples t-test was used to compare the volume of fluid and number of fluid pockets between age-matched fluid-fed and fasted children. Where multiple age-groups were compared ANOVA tests were undertaken.

4.10 Results

MRI data sets were available from 32 fasted children and 23 fluid-fed children. The demographics of the participants are shown in Table 1. The youngest child was one week old and the oldest 16 years old. The gender split was approximately even with 52% (12/23) of the fluid-fed participants and 59% (19/32) of the fasted participants being female. All data sets were of sufficient quality to allow subsequent analysis.

Table 4 2 Demographics of the participants included in the study.

	Number of data sets available	
Age range	Fasted Children	Fluid-fed Children
<2 years (newborn/infant/toddler)	10	
2-5 years (pre-school children)	13	
6-11 years (school age children)	8	6
12-16 years (adolescents)	1	17

4.10.1 Gastric fluid volume

The gastric fluid was either present as a single pocket or absent. Residual gastric fluid was absent in 65.6% (21/32) of fasted children and present in all fluid-fed children. A comparison of the total volume of gastric fluid present in fasted vs fluid-fed children is shown in Figure 4 2. The difference in gastric fluid present in fluid-fed versus fasted children was statistically significant ($p<0.05$).

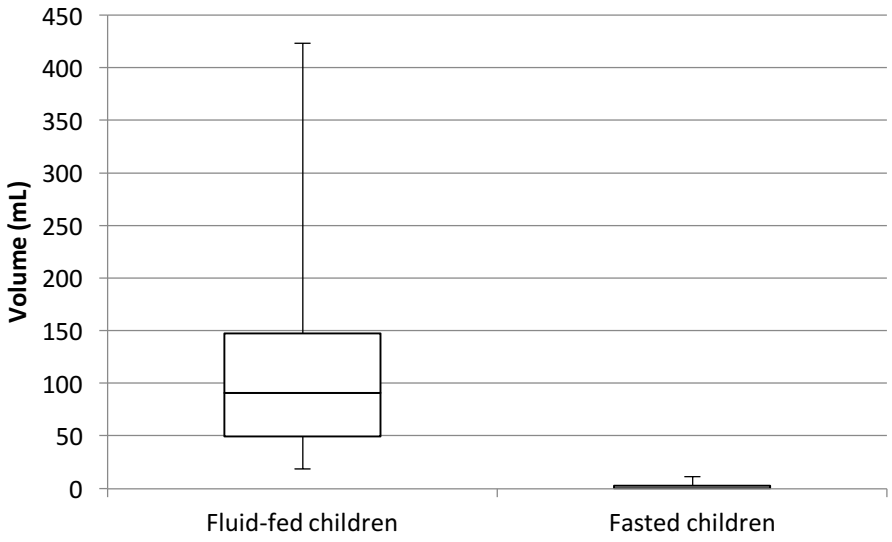


Figure 4 2 Comparison of gastric volumes in fluid-fed (n=23) and fasted (n=32) children. Data shows median; Q1; Q3 and range.

The age of the child had no impact on the volume of gastric fluid present in either fasted or fluid-fed children ($p>0.05$); this data is shown in the supporting information (Figure 4 10).

4.10.2 Number of pockets in the small intestine

The total number of fluid pockets identified in the small intestine of fasted patients ranged from 0-16; whereas for fluid-fed patients it ranged from 11-40, as shown in Figure 4 3.

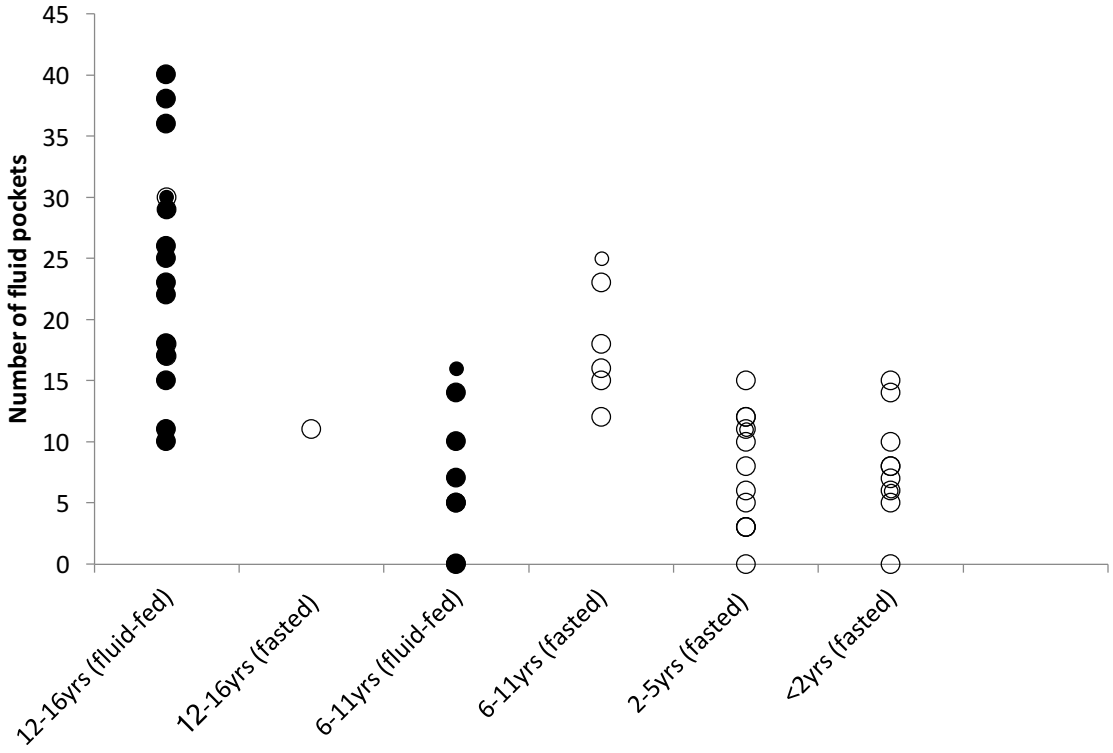


Figure 4 3 Comparison of the number of fluid pockets identified in fluid-fed (n=23) (shown as black circles) and fasted (n=32) (shown as white circles) children. Data is stratified by age group as well as the fluid-fed and fasted state.

As the fluid-fed children were typically older than the fasted children the impact of age on the number of fluid pockets was explored. Participants were stratified into groups based on those <2 years; 2-5 years; 6-11 years and 12-16 years to determine whether there was a difference in the number of fluid pockets identified. The number of pockets in aged-matched (school age children) was significantly different ($p = 0.011$) for fluid-fed (n=6) and fasted (n=8) children. Pooled analysis of all participants showed that there were significant differences in the number of pockets identified based on age for the overall population ($p<0.05$); see supplementary material. However, there was no statistically significant difference ($p= 0.420$) for the number of pockets in fasted children based on their age.

4.10.3 Location of fluid pockets

In both the fluid-fed and fasted populations the majority of fluid pockets were located in the jejunum; 86% in fluid-fed children and 93% in fasted children, all remaining pockets were located in the ileum.

4.10.4 Small intestinal fluid volume

The overall volume of fluid in the small intestine was compared by both age range and the fluid-fed/fasted state. The results are shown in Figure 4 4. The mean total small intestinal volumes were 30 ±24mL and 7 ±10mL in the fluid-fed and fasted state respectively.

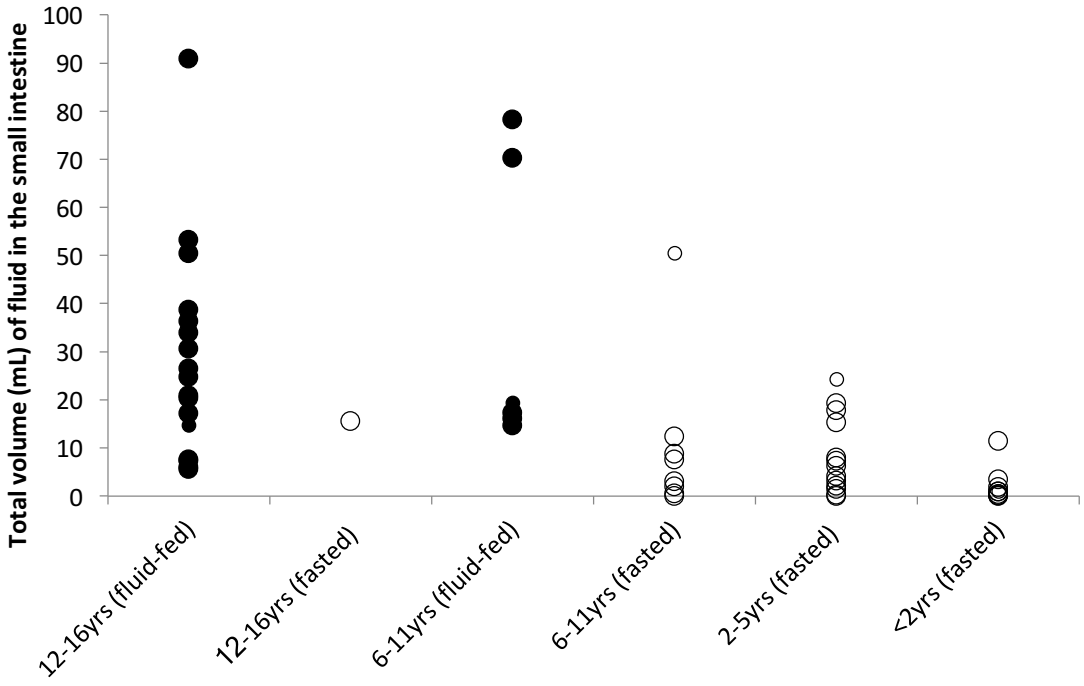
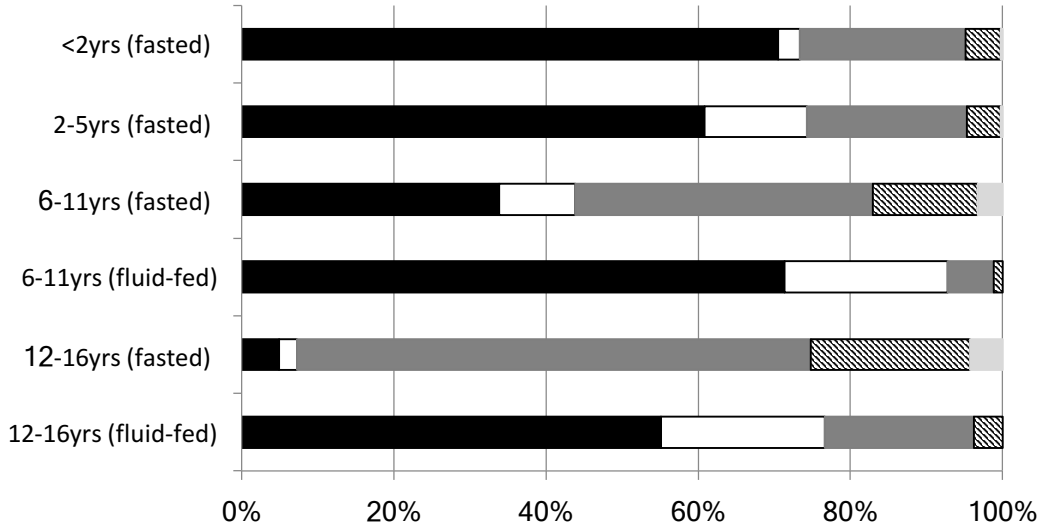


Figure 4 4Total small intestinal volume presented as a function of age range and the fluid-fed/fasted state.

Statistical analysis showed that there is not a statistically significant difference in the total volume of fluid in the small intestine in school age children between the fluid-fed and fasted state ($p=0.101$); although there is an outlier with one fasted participant with a small intestinal fluid volume of 50mL which has skewed the results; elimination of this outlier resulted in a significant difference in the values ($p<0.05$). Age had no significant effect on the total volume for those in the fluid-fed or the fasted state sub-populations.

The proportion of pockets that were less than 1mL was 66.0% (306/464) in fluid-fed children and 76.5% (202/264) in fasted children. These small pockets contributed to 21% of the total fluid found in the small intestine of the fluid-fed participants and 27% for the fasted participants. Figure 4 5 shows (a) the relative number and (b) proportion of fluid present based on pocket size in the intestine of the fluid-fed and fasted participants; this are shown within age sub-populations although no differences were seen based on age.

(a)



(b)

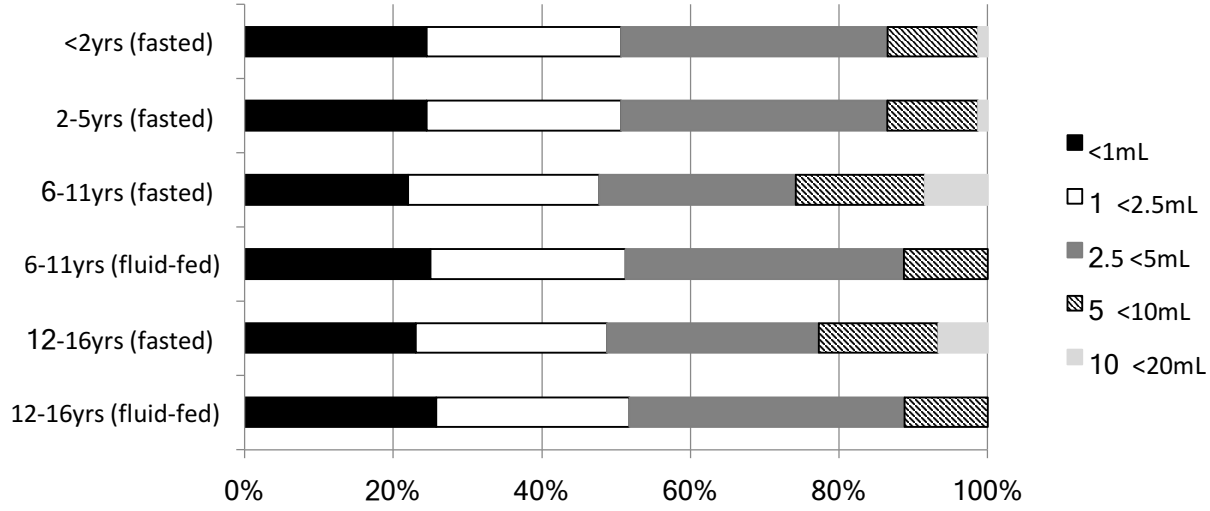


Figure 4 5 Comparison of (a) the percentage of fluid pockets by each volume per sub-population and (b) the total volume occupied by these pockets by each sub-population.

4.11 Discussion

Knowledge of the volume and distribution of gastric and small intestinal fluid in children in both the fluid-fed and fasted state is of great interest to the pharmaceutical industry. This information will inform the design of bio-relevant *in vitro* and *in silico* methods that will predict the performance of oral solid dosage forms which can, in turn, aid in prediction of the pharmacokinetics of a drug. In the current study two populations were available; those who were fasted and those who were fluid-fed having ingested up to 500mL of Klean Prep in the 60 minutes prior to data collection. These two populations enable the impact of fluid on

overall intestinal volumes to be explored although the data does not exactly replicate clinical testing as has been undertaken in adult studies due to ethical constraints surrounding paediatric clinical testing [Mudie *et al*., 2014].

The MRI protocol for fluid-fed participants required children to consume 500mL Oral Klean Prep (a macrogol solution) in the 60 minutes prior to their scan yet this was not monitored thus there is likely to be variability in the total amount consumed and the time frame for consumption prior to data capture. Data is available that details the time that the fluid-fed participants started the study and the time the MRI was conducted which ranged from 14 minutes to 2 hours 35 minutes with a mean value of 68 minutes. There was no correlation with time compared to volume in the small intestine (correlation showed R^2=0.0006) (Shown in Figure 4 9 of supplementary material).

Our results showed that fasted children had gastric volumes ranging from 0 to 8mL with a mean volume of 1.3 ml. The fasted gastric volume in children is very much lower than that reported in adults (see Figure 4 6) which may have consequences on the disintegration and dissolution of medicines given to children in a fasted state. The inter-individual variability in adult fasted gastric fluid volume has previously been reported as having a relative standard deviation of 31% with intra-individual variability relative standard deviation of 23% [Freuhauf *et al*., 2007]. A comparison of the gastric volume reported in this study compared to previous values from adults is shown in Figure 4 6.

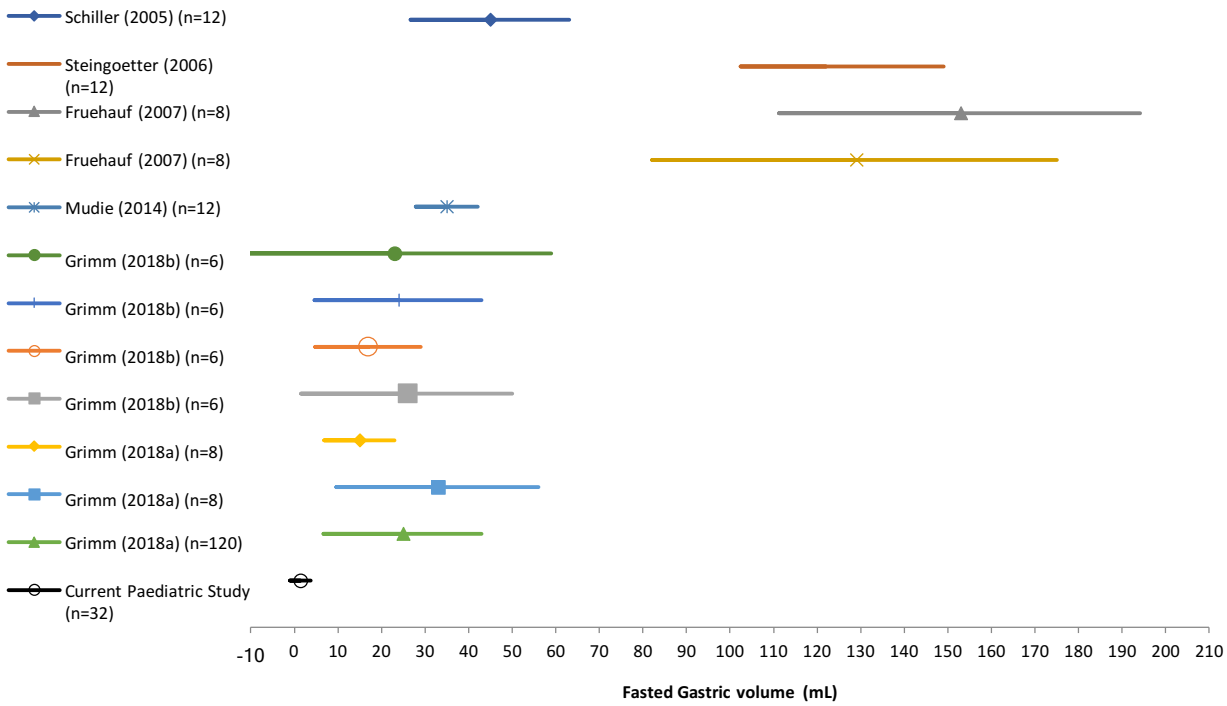


Figure 4 6 Comparison of the mean (± standard deviation) reported for fasted gastric volumes in adult studies compared to the current paediatric study. [Steingoetter study only reported a range not a mean and standard deviation]. Note that several studies included sub-populations hence multiple data from the same source.

The fasted gastric volume reported here is very much lower than that previously shown in adults. Fasting gastric volumes previously reported in children were 0.4-0.56 ml/Kg [Meakin *et al*., 1987; Crawford *et al*., 1990]. The data in this study would provide an estimated value would be 0.08mL/Kg (based on a mean age of 4 years with an approximate weight of 16 Kg). This much lower fasting gastric volume has implications in dosing to children where it is important to highlight the need to dose with water. Oral suspensions are a commonly used dosage form in children where drug dissolution is a rate limiting step for absorption. Previous work has demonstrated that larger gastric fluid volumes enhance dissolution of poorly soluble drugs [Nader *et al*, 2016], therefore low gastric volumes may impact the onset of drug action. Medicines administration in children is often not accompanied by an excess of water, particularly for liquid medications [Hens *et al*., 2017]. Furthermore, there is evidence that children are often on fluid restricted diets prior to night-time dosing which may lead to slow onset of action for medicines given at night [NICE Clinical Guidelines, 2010]. The lowest fasted gastric volume previously reported in adults was 1mL and the highest was 95mL [Grimm *et al*., 2018a]; our results showed that almost two-thirds of fasted children had no measureable gastric fluid volume.

Previous work that has looked at the impact of gastric volumes following ingestion of water have shown increased gastric volumes of 296mL immediately after ingestion of 300mL water [Steingoeter *et al*., 2006] and 242mL following ingestion of 240mL water [Mudie *et al.,* 2014]. Our study collected data from children who had ingested up to 500mL Klean prep in the 60 minutes prior to the MRI; the data showed increased gastric volume yet the data is not directly comparable to that found in adults, due to the differences in protocols used. Ethical restrictions limit the extent of experimentation that can be conducted using MRI with paediatric populations.

This study has confirmed that, as previously seen in adults, fluid is present in discontinuous pockets in the small intestine of children in both the fluid-fed and fasted state. Discontinuous pockets of fluid in the small intestine of adults was first reported in 2005 by Schiller *et al*. The total number of pockets identified in our study was 8 ±4 and 22 ±4 in the fasted and fluid-fed state respectively. This data is similar to that reported by Mudie *et al* where 8 pockets were reported in the fasted state in adults and 16 in the fed state (in adults) [Mudie *et al*., 2014].

A total mean small intestinal volume of 105 mL (range 45-319mL) was reported in the fasted state in a study conducted by Schiller *et al* in 2005. A lower total volume of 43mL (range 5-158mL) was reported by Mudie *et al* [2014] which was similar to a value of 54 ±37mL reported by Grimm *et al* [2018] [Schiller *et al*., 2005]. Our study reported a fasted mean small intestinal volume of 7.4mL (range 0-50.58 mL) in children which is much lower than values previously reported in adults. The forest plot in Figure 4 7 compares the volumes of fasted small intestinal fluid reported in the literature to the values found in our study.

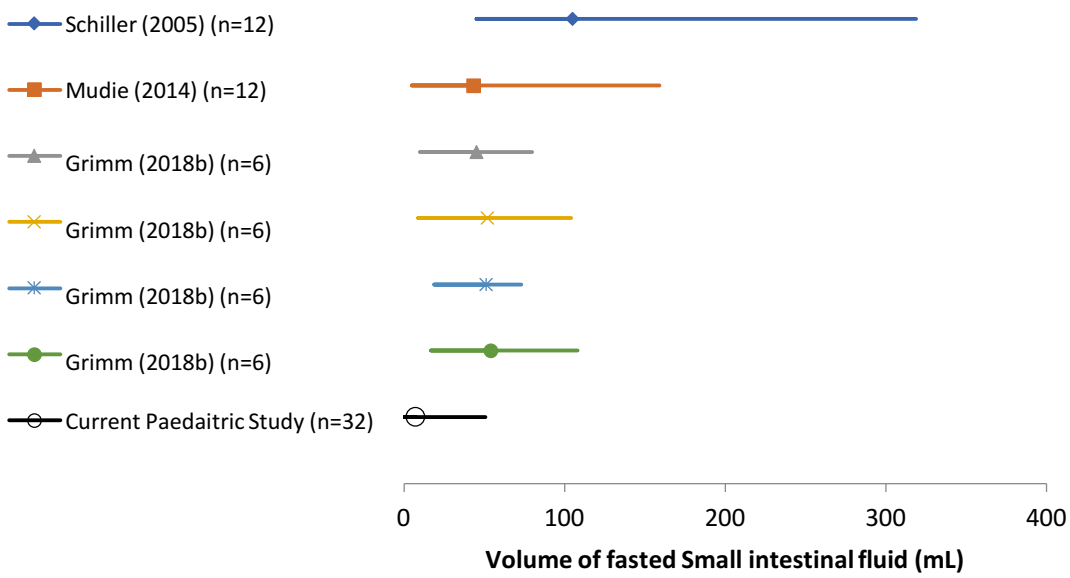


Figure 4 7 Comparison of reported volumes of fasted small intestinal fluid in adult studies compared to the current paediatric study (data shows mean and range except for Grimm (2018b) study where mean ±standard deviation is shown as range data was not available).

This lower volume in children is even smaller than values previously used in representative paediatric dissolution testing. Conventional dissolution testing of pharmaceutical dosage forms typically involves large volumes of fluid (500-900mL). The appropriate volume to be used to represent the paediatric intestinal media has previously been suggested to be 50mL for neonates; 100mL for infants and 200mL for pre-school children [Wollner *et al.*, 2018]. This study suggests that volumes of 7-40mL may be more appropriate in pre-school and school aged children. However, it is important to consider that this volume is not present as a single homogenous pocket but is distributed into several small pockets along the intestinal tract. Figure 4 8 shows a cartoon bubble plot that represents the distribution and relative size of fluid pockets in fluid-fed and fasted children.

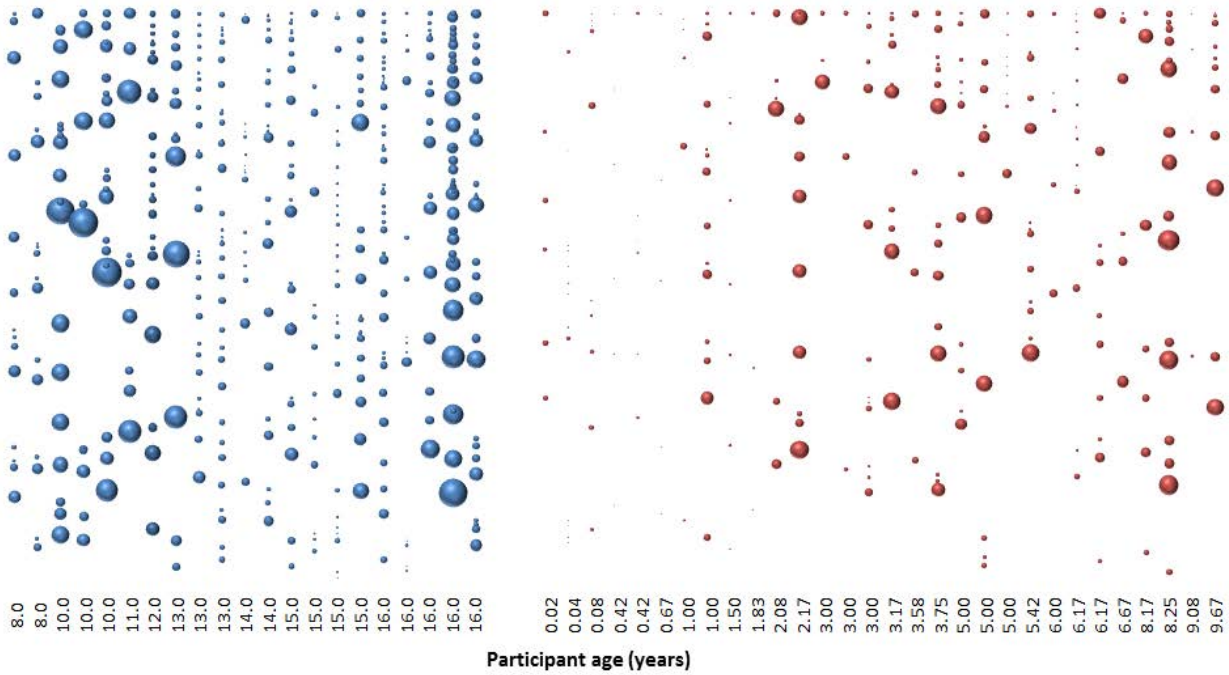


Figure 4 8 Representation of fluid pockets in fluid-fed children (blue) and fasted children (red); each bubble represents one pocket and the bubble size the relative volume. The data is presented from left to right in order of age for each sub-population.

Limitations of our data set need to be considered. The presented data were all collected from children undergoing MRI for clinical reasons, therefore the protocol for ingestion of fluid was not tightly controlled and the actual volume and timing of ingestion of the recommended 500mL of Oral Klean Prep was not accurately recorded for the fluid-fed participants. Adherence to the protocol for fasted children was also not checked where children were asked to fast overnight prior to the MRI. It is recognised that using Klean Prep (an osmotic laxative) to represent fluid fed children is not fully representative of a typical fluid fed child. The mechanism of action of this product is to draw water into the intestines which maximises the fluid content increasing the water content and volume of stools in the bowel. The Klean prep was administered within 60 minutes of imaging thus the fluid present in the intestine was observed prior to defecation. This provided a fluid fed scenario to allow comparisons to the fluid fasted children. The age ranges of the children who were fasted and fluid-fed are not matched and additional efforts are ongoing to try to match the demographics of fluid-fed and fasted children.

4.12 Acknowledgments

The research described in this paper was sponsored by a Ph.D. grant from Janssen Research & Development, A Division of Janssen Pharmaceutica NV.

4.13 Abbreviations

GI, gastrointestinal; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; PBPK models, Physiologically based Pharmacokinetic models; UHCW, University Hospitals Coventry and Warwickshire; BCH; Birmingham Children's Hospital.

4.15 Supplementary information of the paper is available

- Relationship between the total small intestinal volume measured and the time lapsed from instruction to consume fluid to the time of MRI data collection for the fluid-fed participants
- Comparison of gastric volume vs age for fluid-fed and fasted participants

4.15 Supplementary information of the paper

*P*ublished material

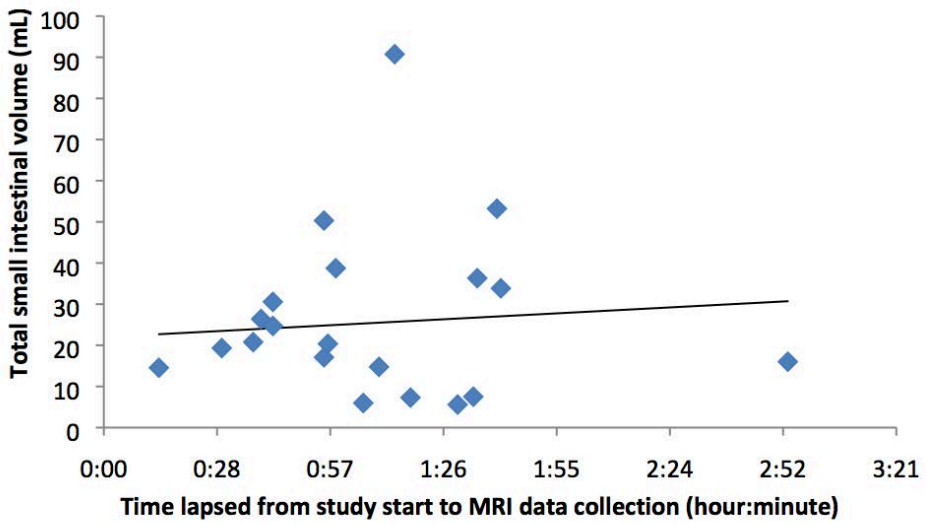


Figure 4 9 Relationship between the total small intestinal volume measured and the time lapsed from instruction to consume fluid to the time of MRI data collection for the fluid-fed participants.

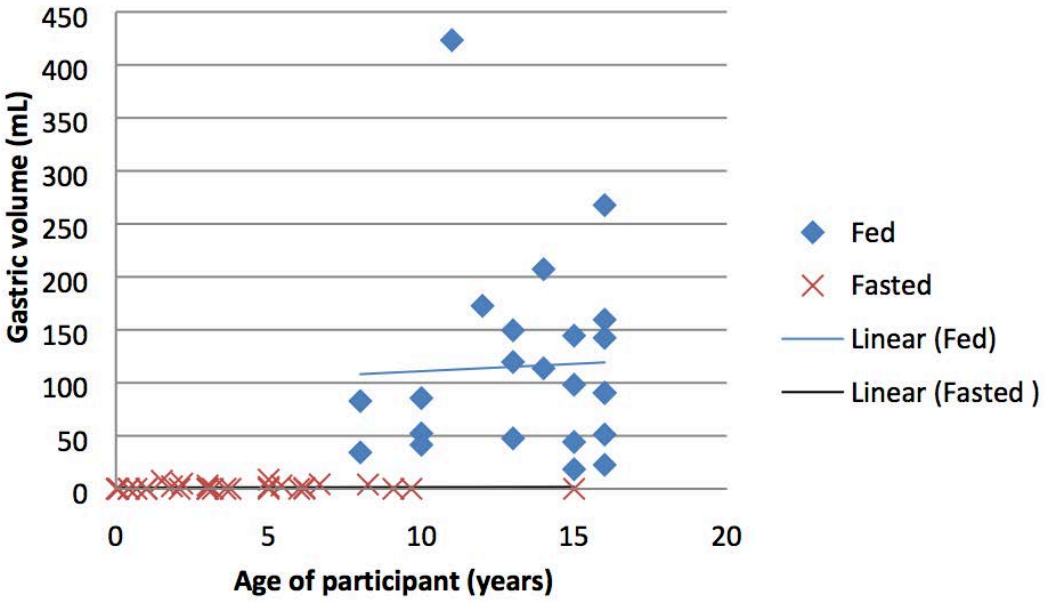


Figure 4 10 Comparison of gastric volume vs age for fluid-fed and fasted participants

4.16 Supporting information to the thesis

The anonymised MRI images obtained were analysed using Horos software which is a free, open source medical image viewer (https://horosproject.org/about/). In addition, ImageJ software was used for quantification of areas and volumes (ImageJ is an open source image processing program designed for scientific multidimensional images, https://imagej.net). Below is presented additional information on image processing, including protocols for both HOROS and ImageJ software and figures that were obtained during image analysis.

4.16.1 Details on image processing

MR Images were obtained by using T2 weighted sequence. In practice, increased water and methemoglobin in subacute haemorrhage appear bright on T2- weighted image. Low proton density, calcification, fibrous tissue, paramagnetic substances and protein rich fluid are presented as dark signals on T2- weighted image. On the other hand, increased water, as in oedema, infarction, inflammation and infection, low proton density and flow void appears dark on T1-weighted image. Furthermore, fat, subacute haemorrhage, melanin, protein-rich fluid, slowly flowing blood are presented as bright signals on T1- weighted image [Johnson, 2017].

In terms of fluid volume calculations, regions of fluid pockets were identified via HOROS software after comparison with cerebrospinal fluid (CSF) and gall bladder as internal calibrators. The process is presented at 4.16.1.1 MRI Images Analysis Protocol below. 4.16.1.2 ImageJ analysis protocol was used to generate data on fluid pockets, and their surface area values. Next, the total fluid volume was measured with calculations based on the slice-gap thicknesses between the slices, a parameter unique for each dataset. Further details on this process is outlined below.

4.16.1.1 MRI Images Analysis Protocol

4.16.1.1.1. Importing files:

Option *import* was selected from the toolbar. Then, one or more files were copied to the dataset. Alternatively, the user could simply *drag* and *drop* the files of choice to the dataset.

4.16.1.1.2. Selection of the regions of interest (ROIs)

Prior to calculating the ROIs, it should be made clear how many slices out of total the small intestine is visible. The first step is to divide the GI tract in four major quadrants: Right upper quadrant (RUQ), right lower quadrant (RLQ), right upper quadrant (LUQ)

and left lower quadrant (LLQ) (Figure 4 12) A square is designed with the rectangle tool so as to include the GI tract in it. This is fundamental for the accurate determination of the localisation of the ROIs. The upper and the lower limits are set to be horizontal hypothetical lines from the top of the liver and the bottom of pelvis respectively. Additionally, the spine can be used as a vertical axis for the GI tract. Furthermore, mesentery and blood vessels must be excluded from the ROI when calculating the total area. In order to ensure the location of the small bowel, the large intestine must be first identified. Prior to selecting the ROIs, magnification of the image must be done to achieve a more detailed view of the image. Via using the Global Threshold plug-in tool, the software can identify all signal regions within the GI tract with intensity above a threshold, which is determined by the subject's cerebrospinal fluid. The process is described in detail below at section "

4.16.1.1.3 Signal Thresholding". The surface area of the ROIs is calculated automatically by the software. In order to separate the signal regions in the small intestine from the rest of the G.I tract, they were drawn with the closed polygon tool. Dots were created spherically around the ROI, outside its outline. Once the dots are automatically connected together, the area of the ROI is calculated by the software in square centimeters. For adapting the spherical shape more closely to the ROI, the repulsor tool can be used. Repulsor tool appears as a circle that can smoothly "push" the drawn line around the ROI. The ROI can be saved by selecting ROI option at the toolbar and then by selecting "Save selected ROI" tool. Figure 4 11 below is an example of how the MR images appear after thresholding based on the CSF.

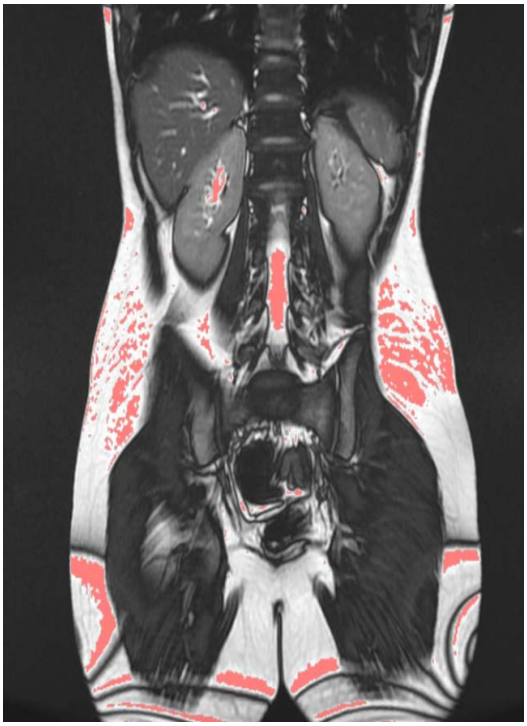


Figure 4 11 Determination of threshold based on patient's CSF.

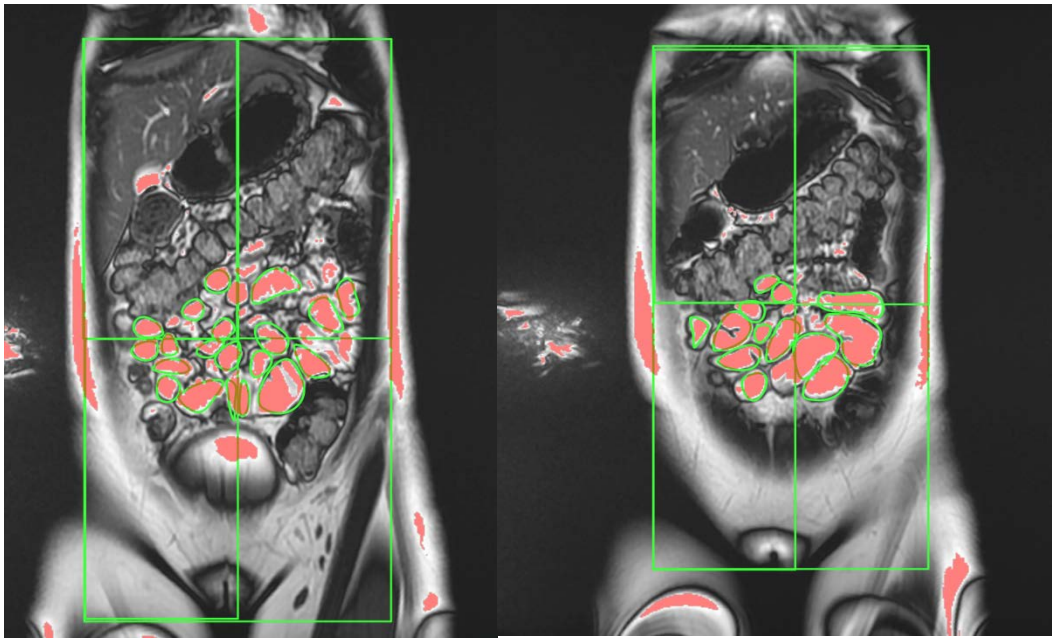


Figure 4 12 MR sequence showing MR image after thresholding and division to the four major quadrants: Right upper quadrant (RUQ), right lower quadrant (RLQ), right upper quadrant (LUQ) and left lower quadrant (LLQ).

4.16.1.1.3 Signal Thresholding

The MRI data were thresholded in a way to include intra and inter subject signal variations. Internal calibration of signal intensity levels via the Cerebrospinal fluid (CSF) was applied. The Gall bladder was not appropriate for conducting the calibration, as it would empty and refill

over the course of image taking for subjects that ate a “complex meal containing fat”. Additionally, the gall bladder contains cholesterol which would reduce the quality of intrinsic standard signal [Hoad *et al*, 2007].

The method hypothesizes that any pixel in the GI tract with a signal intensity higher than a known threshold (S_{th}) is filled with water [Hoad *et al*, 2007]. It is worth mentioning that this threshold must be estimated for each MRI data set, as scanner instabilities, subjects repositioning and coil loading can affect the S_{th}.

4.16.1.2 ImageJ analysis protocol

The files open as following at ImageJ: File → open → select image.
Then, the measurement scale was set by drawing a line along the ruler from the image ruler then selecting Analyze → Set Scale. 10 cm was the value entered in set scale window into the the 'Known Distance' box. Additionally, the 'Unit of Measurement' box was changed to cm, confirming that the 'Global' option is selected. To verify that the measurement scale was correct, a line was drawn. Next, the image was converted to a 16- bit image by selecting image → type → 16 bit. This step converts the image to black and white as seen in Figure 4 13

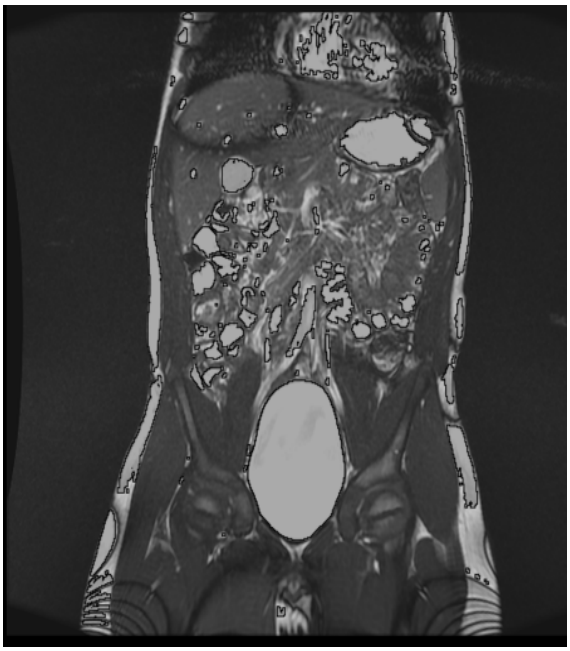


Figure 4 13 MR image converted to 16 bit via ImageJ.

At this step, the threshold was adjust as following: Image → Adjust → Threshold

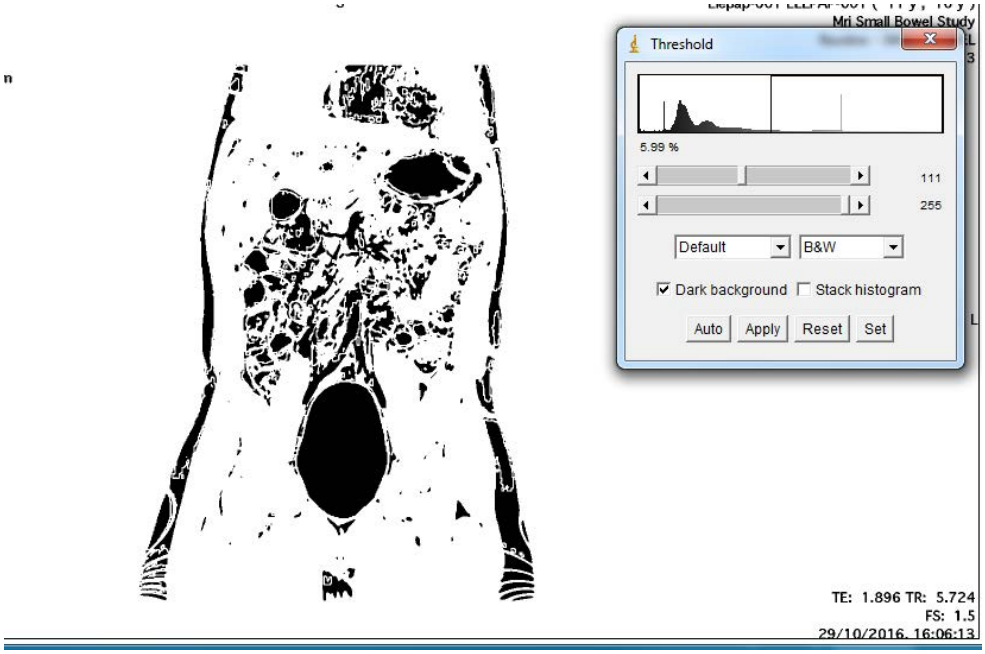


Figure 4 14 ImageJ step allowing to adjust the scales to define the region of interest and also changes the image to a negative

Next, a rectangle was drawn around the region of interest and the operator clicked on analyse and then on analyse particles (Figure 4 15).

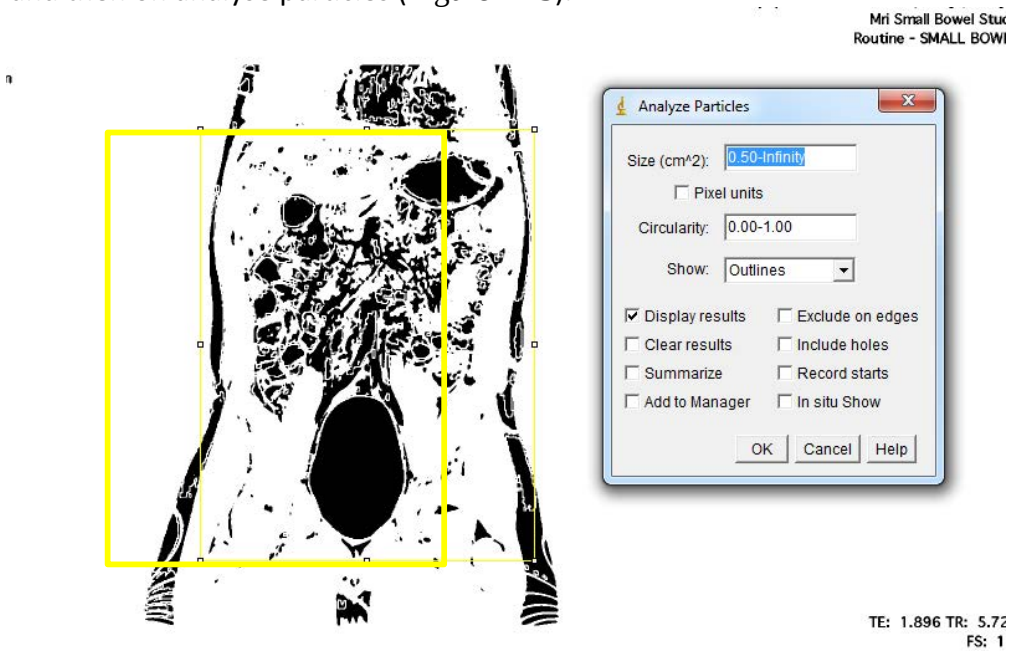


Figure 4 15 Image generated after selecting the "analyse particles" option. The yellow frame is drawn around the area of interest to limit the particles analysis.

A table (

Figure 4 17) showing the surface areas' values in cm^2 is generated together with the final figure, which demonstrates the outlines of the fluid pockets (Figure 4 16).

Figure 4 16 Final image generated by ImageJ

Results

File Edit Font Results

	Area	Mean	Min	Max
1	1.110	157.582	111	194
2	12.472	167.345	111	172
3	1.194	163.816	111	172
4	1.253	139.116	111	177
5	6.392	164.360	111	172
6	3.217	149.199	111	159
7	4.474	148.080	111	201
8	1.355	135.165	111	196
9	6.279	141.414	111	199
10	1.032	135.710	111	189
11	0.576	150.918	111	170
12	0.717	137.243	111	203
13	0.662	132.243	111	175
14	1.975	166.233	111	172
15	2.788	146.675	111	202
16	4.810	141.427	111	201
17	1.764	133.270	111	198
18	1.194	163.290	111	172
19	2.081	165.161	111	172
20	1.594	141.672	111	200
21	2.884	162.830	111	173
22	0.507	131.131	111	190
23	2.651	149.835	111	170
24	0.672	161.650	111	172
25	1.365	141.131	111	204
26	0.628	132.408	111	184
27	0.838	159.832	111	171
28	0.656	157.212	111	171
29	3.346	147.339	111	208
30	0.570	162.309	111	171

Figure 4 17 Table generated by ImageJ

5 Chapter 5: Physiological Based Pharmacokinetic Model (PBPK) Model

Paediatric physiologically based Pharmacokinetic (PBPK) model chapter

5.1 Relevance to thesis

This PBPK chapter integrates the values of small intestinal fluid present in children that was obtained using MRI data (chapter 4). Based on the data within the MRI chapter, it was noted that the actual volumes present in the GI tract of children was lower than previously considered and there was a need to update the existing fasted and fed paediatric populations within PBPK software. Updated small intestinal fluid volume data have been inserted into the SimCyp paediatric model, in an attempt to observe the impact of the change in fluid on the predicted pharmacokinetic profile of a model drug. SimCyp software is a commonly used and established PBPK modelling software.

5.2 Introduction

5.2.1 Development of PBPK model

Physiological based pharmacokinetic (PBPK) models are computer modelling approaches that link together the different body compartments of the body, the tissue composition of the organs and the blood flow to predict the pharmacokinetics (PK) of drugs. The PBPK model concept was initially introduced by Teorell in 1937 [Teorell, 1937]. PBPK models work by incorporating a drug's information with knowledge on the physiology and the biology of a selected patient's group in order to achieve a representation of the drug's behaviour in biological systems [Zhuang and Lu, 2016].

The following decades, there have been efforts on improving and updating the PBPK modelling so they could play a pivotal role in drug development [Raddy *et al.,* 2005]. The advances in computer functionality had a domino effect on PBPK software development. However, it was only 2011 when PBPK models have been significantly accepted by the regulatory agencies and started playing a pivotal role in drug development and regulatory assessment [Zhao *et al*., 2011; Rostami- Hodjegan *et al*., 2012]. Nowadays, PBPK models hold a key role not only during drug discovery and development [Rowland *et al*. 2011; Jones *et al*., 2015] but also in the regulatory process [Sinha *et al*., 2014; Wagner *et al.,* 2015].

In general, PBPK and PBPK-PD (modelling and simulation) M&S are characterized by a 'bottom up" modelling of biological compartments that are combined together to form larger

subsystems, which are usually linked together to create an even larger and complete multiscale model. When working on a PBPK model, the first task is to identify and integrate the physiological data into the systems and next, coupling these with clinical data to show verification.

5.2.2 Structuring a paediatric PBPK model

A PBPK model consists a multi-compartment model, where each compartment represents a different organ in the body [Barrett, 2012]. "Mass-balance" equations describe drug appearance in each organ, from when it enters the arterial blood to when it exits the venous blood. Most commonly, when developing a paediatric PBPK model, a PBPK model that has been validated with adult data is used and the differences in growth and maturation, that can affect anyhow the drug disposition and pharmacodynamics (PD), are incorporated [Barrett, 2012]. Figure 5 1 demonstrates how the patient factors and the PBPK model components are combined to build an accurate PBPK model.

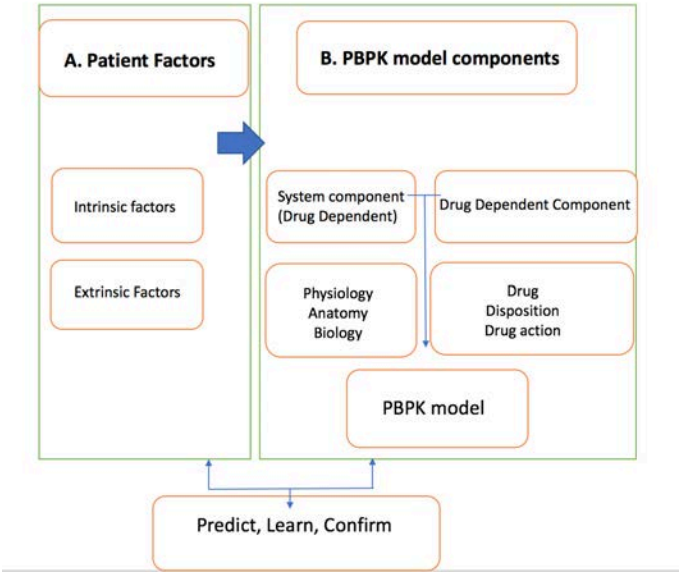


Figure 5 1 Graph of structuring process of a PBPK model. Table adapted from Huang and Temple, 2008.

It has been previously discussed and acknowledged that the age developmental changes that occur from infancy to adolescence are massive (2.10. Paediatric Gastrointestinal fluid composition) and therefore, predicting the drug response for each age group separately is of importance. Extrapolating pharmacokinetic data from adults to paediatric populations has

proven to be an inaccurate practice, leading to under-prediction of drug-drug interactions [Mahmood, 2014].

The major developmental changes that are crucial to drug pharmacokinetics are summarized in Table 5 1

Table 5 1 Maturation and growth changes affecting drug pharmacokinetics. Table adapted from Barrett *et al.* [2012].

DRUG ABSORPTION [Kearns *et al*., 2003]	**DRUG DISTRIBUTION** [Kearns *et al*., 2003]	**HEPATIC METABOLISM** [Icorn and McNamara, 2002; Johnson *et al*., 2006]	**RENAL EXCRETION** [Icorn and McNamara, 2002; Johnson *et al*., 2006]
Changes in gastric emptying and intestinal transit time	Changes in body fluid compartments (e.g total body water in neonates: 78% vs adults: 55% etc.)	Liver size is relatively bigger in infants/ children than in adults	Glomerular filtration rate is less in infants/ children than in adults
Ph Changes in intestinal segments	Body fat percentage is lower in children than in adults	Drug metabolism enzymes are affected by developmental changes	Renal tubular absorption and secretion are less in infants/ children
Changes in intestinal transporters and enzymes that cause first pass metabolism	Protein binging is less in infants/ children than in adults		
	BBB is more permeable in infants/ children than in adults		

Several PBPK models have been developed by multiple research groups attempting to integrate as many maturation and growth aspects as possible [Bjorkman, 2005; Mouksassi *et al*., 2009; Edginton *et al.,* 2006; Johnson *et al*., 2006; Ginsberg *et al*., 2004]. Common practice is that PBPK modelling and simulation (M&S) researchers use pharmacokinetic data sets either from literature or from their own research for validating these models for the different age groups.

5.2.3 Examples of successful application of PBPK models

Paediatric PBPK models have been used successfully for predicting PK differences across adults and children for various active pharmaceutical ingredients (APIs). For instance, the disposition of theophylline and midazolam is predicted by PBPK model in infants and children [Bjorkman, 2005] Additionally, 11 drugs' variability and clearance are predicted as well (oral and intravenous midazolam, caffeine, carbamazepine, cisapride, theophylline, diclofenac, omeprazole, S-warfarin, phenytoin, gentamicin, and vancomycin, in both infants and children [Johnson *et al*., 2006].

Edginton *et al* [2006] group has extended previously adult PBPK models [Price *et al*., 2003; Willmann *et al*., 2007] to be used for children. A generic PBPK model for paediatrics has been developed, to reflect the changes in children from infancy to adolescence. It was shown that this model predicted with reasonable accuracy the plasma PK profiles of acetaminophen, alfentanil, morphine, theophylline, and levofloxacin [Barrett, 2012].

As expected, it is becoming more and more common to use PBPK approaches for predicting paediatric dose regimens. The US Food and Drug Administration (FDA) has broadly used PBPK M&S for multiple purposes such as deciding about specific clinical pharmacology studies, evaluating study designs and including the most appropriate language in drug labels [Zhao *et al*., 2011].

5.2.4 Limitations of paediatric PBPK modelling

It is beyond question that PBPK application in children is currently expanding. However, there are still some limitations remaining that need to be overcome to provide optimal predicting results [Barrett, 2012]. A limiting step has been the ability of PBPK models to reliably predict plasma concentrations and PK parameters after oral drug administration to different paediatric age groups [Barret, 2012] The PBPK model's prediction's accuracy for children is further complicated by the fact that the GI tract undergoes continuous development. Data regarding these changes are limited [Johnson and Thomson, 2008]. As a result, the PBPK models may lead to biased predictions of bioavailability in paediatric populations.

Interestingly, it has been observed that fed bile salts concentrations could conceal the age-related changes. Subsequently, no clear difference could be detected between adults and children. As a result, the adults' fed bile salts values are being used in the PBPK softwares [Johnson *et al.,* 2018].

The potential of age- related changes occurring in the intestinal permeability has been greatly ignored by the PBPK softwares, despite the fact that several publications have discussed the importance of this issue [Heiman, 1980; Mirochnick *et al.,* 1988]. These age- related changes of the intestinal permeability could be attributed to the villous structure (thickness and width), the circular folds of the small intestine (large flaps of circular shape projecting into the interior part of small intestine), and the pore size of the small intestine.

5.2.5 SimCyp Software

Of several commercially available PBPK/ PD softwares, the SimCyp Population based Simulator is well established across major pharmaceutical companies [Jamei *et al.,* 2013a]. Throughout the years of SimCyp software development, the software has been transformed from a mechanistic drug- drug interaction tool to a complex and sophisticated Model Based Drug Development (MBDD). SimCyp software releases updates every year that incorporate new features that give insight to different population subgroups with the fullest possible details.

5.3 Aim of the study

Prediction of the performance of oral medicines in a paediatric population is complex due to the lack of knowledge of the GI anatomy and physiology in children. To date, the paediatric GI fluid volumes in physiologically based pharmacokinetic (PBPK) models were extrapolated from adult data. Papadatou Soulou *et al*. [2019] (chapter 4) reported measured values of the luminal volumes for fasted and fed paediatric populations. The new data have been integrated within in SimCyp paediatric model v.18 to enhance the accuracy of the model. A model drug was used to evaluate the impact of this new data on the predicted pharmacokinetics. Ritonavir was selected as the model drug as it is known to be poorly soluble and there are existing data on ritonavir demonstrating the validity of existing PBPK models.
Ritonavir is a well-known inhibitor of the enzymes CYP3A4 and CYP2D6 enzyme pathways. It is an antiretroviral drug, and is commonly used in treating HIV/AIDS [Arora *et al*., 2020]. It is frequently used in evaluating the drug- drug interactions of medication that follow that are being metabolised through the CYP3A4 and CYP2D6 pathways. Additionally, it has clinical application as pharmacokinetics booster, as it works by increasing the exposure of CYP3A4 substrates [Arora *et al.,* 2020]. Ritonavir has been well predicted using SimCyp by several groups [Wagner *et al.,* 2017; Arora *et al.,* 2020; Fiolka *et al.,* 2020]

In addition, there was data on ritonavir PK in paediatric populations that enable the accuracy of the modelled data to be evaluated [Chokephaibulkit *et al*., 2002]
The aim of our study was to incorporate the intestinal fluid volumes (mL) for children and to verify the generated fluid volume data by using a commercial PBPK model to explore the predicted pharmacokinetic profile of Ritonavir as a model drug.

5.4 Methods

SimCYP Absorption and metabolism (ADAM) PBPK software (v.18) was used. The drug that was chosen to be used was ritonavir, as this active pharmaceutical ingredient (API) has been thoroughly studied by SimCyp team and a verified compound file exists within the SimCYP software [Wagner *et al*., 2017; Arora *et al.,* 2020; Fiolka *et al*., 2020]. The first step was to identify literature that described the parameter values for ritonavir compound file that is required for the simulation. The inputs for ritonavir were identified from the literature. This was done using the keywords: *simulation, ritonavir, parameters, PBPK models*. The identified paper that included all the required information for structuring a PBPK model was Umehara *et al*. [2018]. The parameters are summarized in the table below [Table 5 2].

Table 5 2Inputs of Ritonavir substrate compound file

Parameter	Value	Reference
Molecular weight (g/mol)	720.944	Drugbank
LogP	4.3	Shebley *et al*. [2017]
Compound type	Monoprotic Base	Colbers *et al*. [2016]
Pka1	2.0	Shebley *et al*. [2017]
B:P ratio	0.6	Shebley *et al*. [2017]
Fu	0.02	Wagner *et al*. [2017]
Absorption model	ADAM model	N/A
Distribution	Minimal PBPK model	N/A
Vss (L/kg)	0.4 (estimated) Method 1/ SimCyp	Colbers *et al*. [2016]
Clearance (L/hr)	13.0	Zhang *et al*. [2013]
Renal clearance (L/hr)	0.27	SimCyp compound library

LogP is the partition coefficient of a molecule between an aqueous and lipohilic phases. It is used as measure of lipophilicity;
Pka1 is used to demonstrate the strength of an acid;
B:P or Blood to plasma ratio is the ratio of concentration of drug in whole blood
Fu is the unbound fraction of the drug
Vss stands for volatile suspended solid L/kg

The small intestinal volume was measured to be 0-51mL in the fasted and 6-91mL in the fluid-fed state [Papadatou Soulou *et al*., 2019]. These intestinal volume values were included into

the paediatric model. A simulated study was conducted with participants aged 0-16 years, while the dosing regimen was a single immediate release 100mg Ritonavir tablet. These parameters were selected to match the clinical data available from [Chokephaibulkit *et al.,* 2002] that was used to compare the predicted data.

5.5 Materials

The Simcyp Simulator (Version 19, Release 1; Certara UK Limited) with its Advanced Dissolution, Absorption and Metabolism (ADAM) model coupled with full body PBPK was used to simulate the absorption and systemic exposure of ritonavir from the commercial formulation Norvir 100 mg tablet in the fasted and fed state. The model was further verified using independent clinical exposure studies reported for the multiple dose administration of 100 mg Norvir Tablet.

5.5.1 PBPK model development

Table 5 3 Ritonavir model compound

Parameter	Value	Source
Molecular weight (g/mol)	720.944	Drug Bank
Log P	4.3	Shebley *et al*., 2017
Compound type	Monoprotic base	Colbers *et al*., 2016
pKa1	2.0	Shebley *et al*., 2017
B:P ratio	0.6	Shebley *et al.,* 2017
Fu	0.02	Wagner *et al*., 2017
Absorption model	ADAM model	
CaCo-2 (Papp: cm/s)	4.788 X 10^{-6}	Profit *et al*., 1999
Distribution	Minimal PBPK Model	
Vss (L/kg)	0.40 (estimated) Method 1- Simcyp	Colbers *et al*., 2016
Clearance (L/hr)	13.0 (estimated)	Zhang *et al*., 2013
Renal clearance ((L/hr)	0.27	Simcyp compound Library

In order to build a robust, dynamic PBPK model for ritonavir, the default values of the model were replaced with data from the Papadatou Soulou et al. (2019) study. This was done to allow a comparison between the default and the new data (Table 5 4). The overall mean fasted small intestinal values obtained from our previous research (Papadatou-Soulou *et al.* 2019) were 0-50.6 mL with coefficient of variation values >50%. These intestinal volume values were included into the paediatric model.

The default values for the small intestinal fluid in both the fed and fasted state are shown in Table 5.4. It can be observed that the volumes are not altered by the fed state in the default system. However, it should be noted that the software will account for the volume of food ingested within the simulation. It was not possible to change the volume of the duodenum within the software. It is observed that the jejunum sections are increased and the ileum sections are decreased to simulate the updated SimCyp model.

Table 5 4 Fasted and fed default and updated intestinal volume values for the ritonavir SimCyp mod

	Duodenum	Jejunum I	Jejujum II	Ileum I	Ileum II	Ileum III	Ileum IV
Default **FASTED**	34.4	21.2	21.1	12.6	12.6	12.6	12.6
Default **FED**	34.4	21.2	21.1	12.6	12.6	12.6	12.6
New Data **FASTED**	34.4	43	43	3.5	3.5	3.5	3.5
New Data **FED**	34.4	46.5	46.5	1.7	1.7	1.7	1.7

5.6 Results

The regional absorption along the GI tract and systemic concentration in plasma were of the greatest interest in terms of output from the simulated clinical trial.

Figure 5 2 shows the predicted absorption in the sections within the small intestine. The data shows that the fraction of the absorbed dose was elevated in the jejunum and ileum for the updated SimCyp model in the fasted paediatric population.

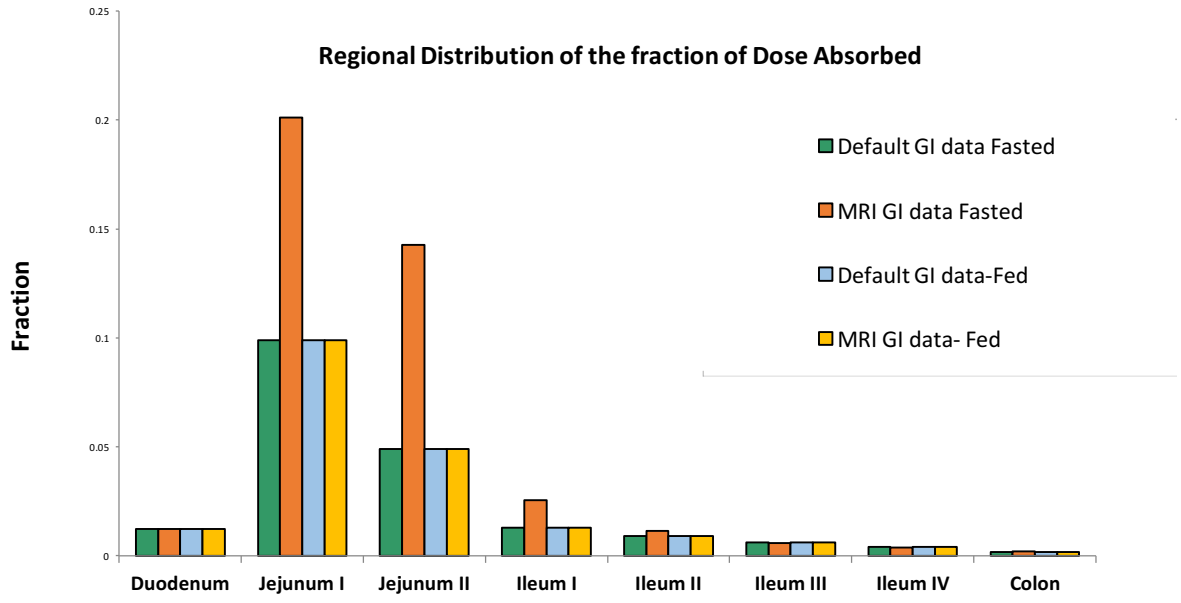


Figure 5 2 Regional Distribution of the fraction of Ritonavir Dose Absorbed by the Paediatric Intestinal Tract as predicted via SimCyp Paediatric.

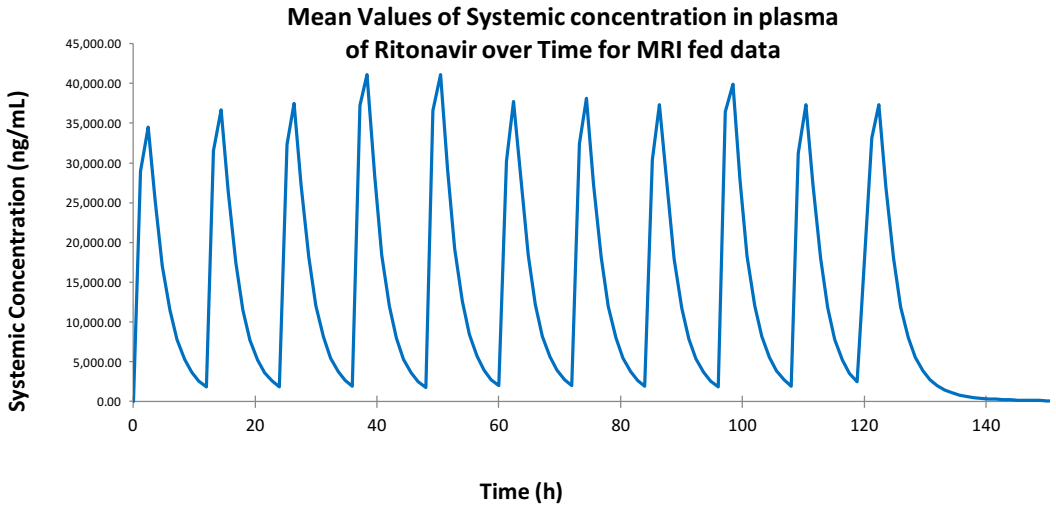


Figure 5 3 Mean values of Systemic Concentration in plasma of Ritonavir for MRI updated values [Papadatou Soulou *et al.*, 2019]

Figure 5 3 demonstrates the mean values of systemic concentration in plasma of ritonavir, using the MRI updated values of Papadatou Soulou *et al*. [2019]. It is observed that they systemic concentration does not reach steady state. Additionally, the peaks are slightly different shapes. This is due to the number of data points; the data in this figure has been smoothed, leading thus to artefacts. It is recognised that a different simulation with increased output would have given more symmetric peaks, however the generated image is a balance in processing power and output.

Figure 5 4 shows the predicted PK profile in the fasted state using the default GI fluid volume values compared to the updated values. The figure also shows clinical data from the Chokephaibulkit *et al*. [2002] study, as the orange dots. It can be seen that the Cmax value of the updated model is a better fit to the clinical data and somewhat lower than the default data. However, it is also to be noted that neither model accurately predict the elimination phase of ritonavir.

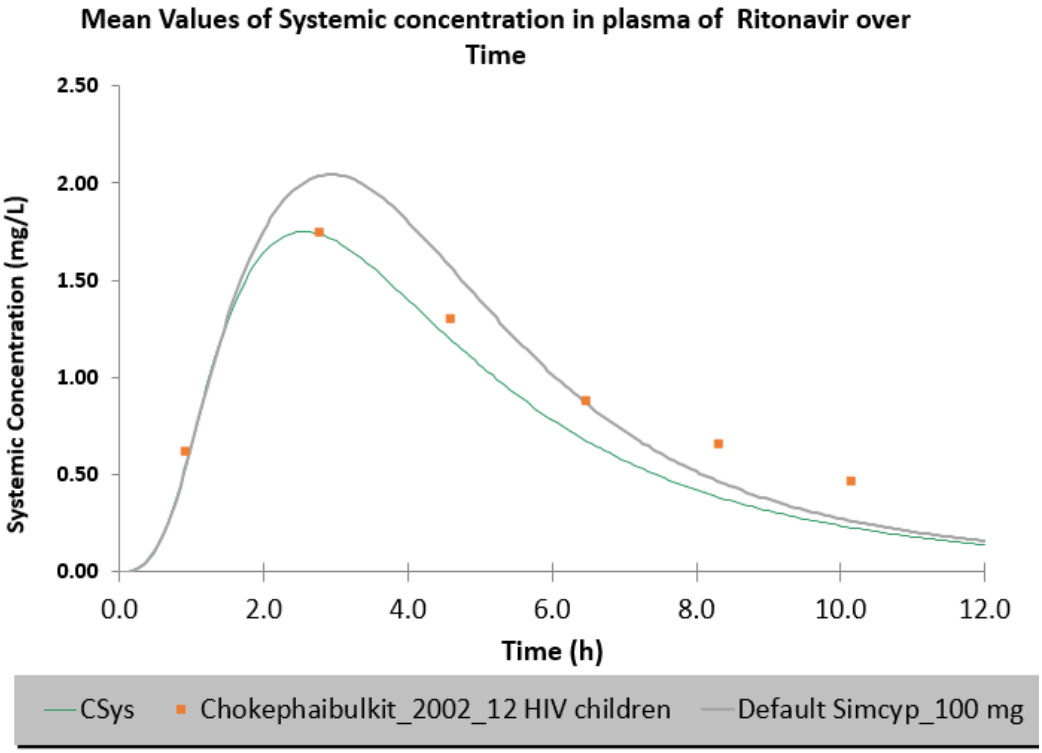


Figure 5 4 Model Verification using data from HIV infected children (orange dots). Dose 100 mg soft gel capsules twice a day. The data points show the observed data and the green line the predicted concentration using the new GI volumes [CSys: Systemic Concentration]

5.7 Discussion

From the graphs obtained from the updated paediatric model it is evident that the fraction of the dose absorbed in the jejunum section is elevated in the new model although there is overall less absorbed compared to the default paediatric population model for the fasted

population. The new paediatric model has been compared to clinical data from a study conducted on HIV infected children, receiving ritonavir treatment [Chokephaibulkit *et al.,* 2002]. The developed model shows good fit to ritonavir as model drug. It is clear from Figure 5 2 that the updated values result into different regional distribution across the intestinal tract. For most drugs, the maximum absorption takes place at small intestine [Nicolas *et al*., 2017]. Thus, it is expected that the observed changes in the dose fraction would have an impact on drug absorption.

It is suggested that the new fluid volume data should be taken into consideration when developing PBPK models for prediction, as paediatric population is a very complex group. It is obvious that better understanding of their anatomy and physiology will enhance the prediction of absorption not only for ritonavir, but for each medication that is absorbed by the small intestine and thus, will improve the current paediatric therapies.

5.8 Supporting information

SimCyp protocol: The simulation step by step

Using as a reference a paper of Umehara *et al.* [2009], ritonavir values were used when inserting substrate details. To start with, population was selected to be: paediatric. The left icon to the substrate should be selected, and details will be adjusted accordingly. Then, population icon to the left was selected and the details were adjusted according to our new, paediatric MRI data.

After that, the trial design button to the left was selected. Details, as how many trials will run, the target population etc. were selected.

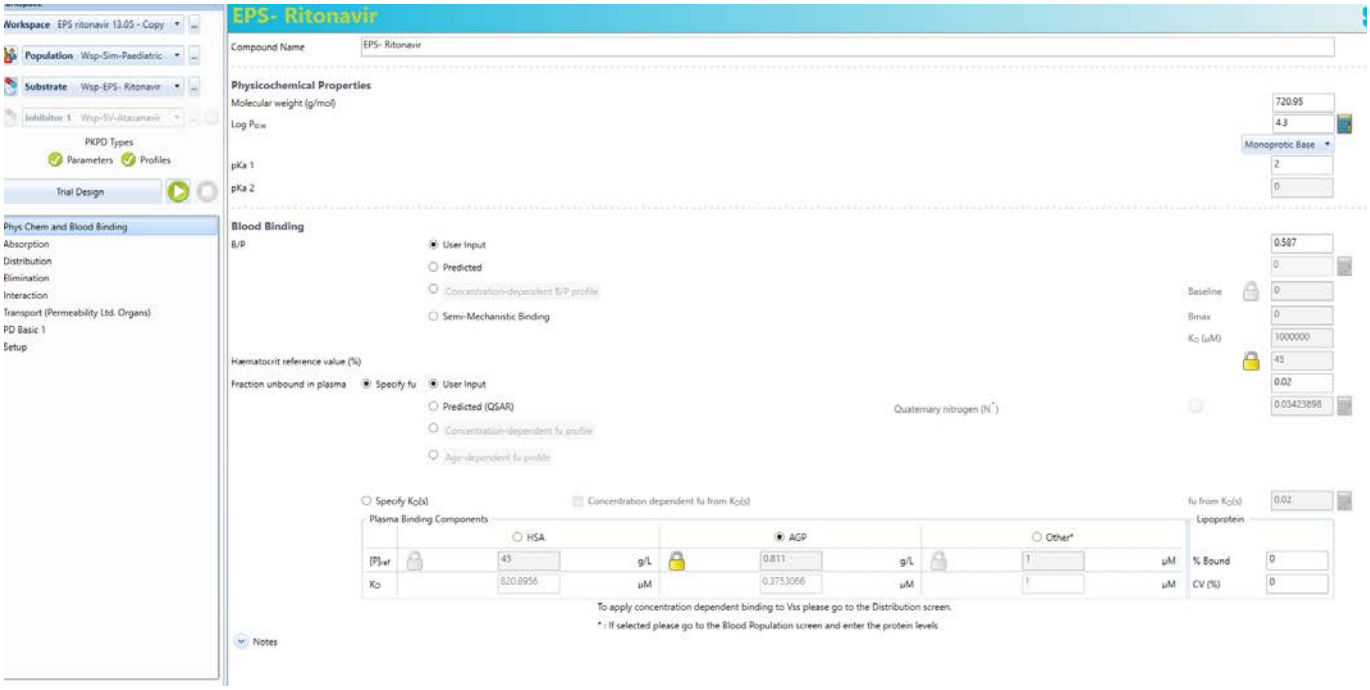


Figure 5 5 Physicochemical and blood binding option, where the required parameters are filled according to Umehara *et al*. [2009] paper.

It is crucial to correctly fill the absorption, distribution and elimination tabs (Figure 5 6, Figure 5 7, Figure 5 8), underneath the physicochemical and blood binding option, in order to have both accurate simulation and graphs.

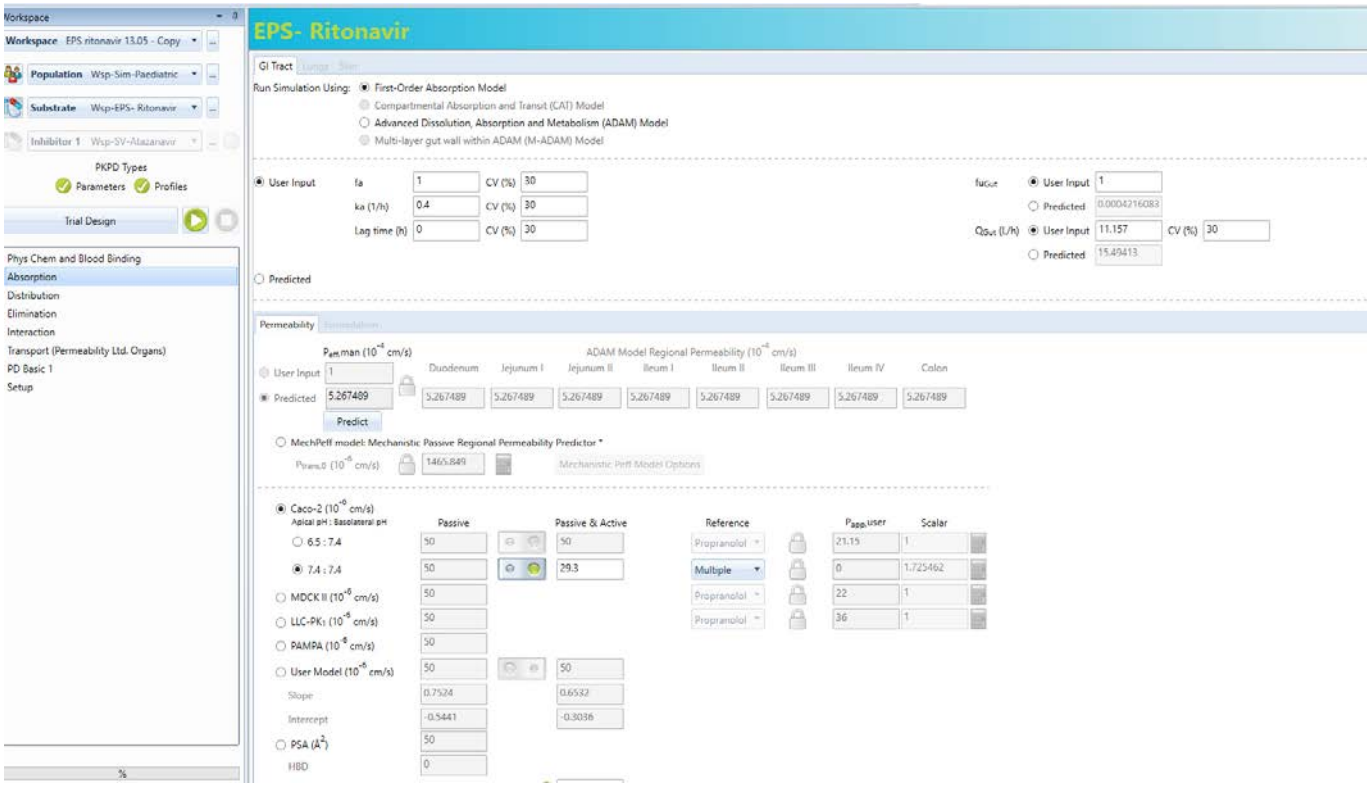


Figure 5 6 Filling the absorption tab with ritonavir details

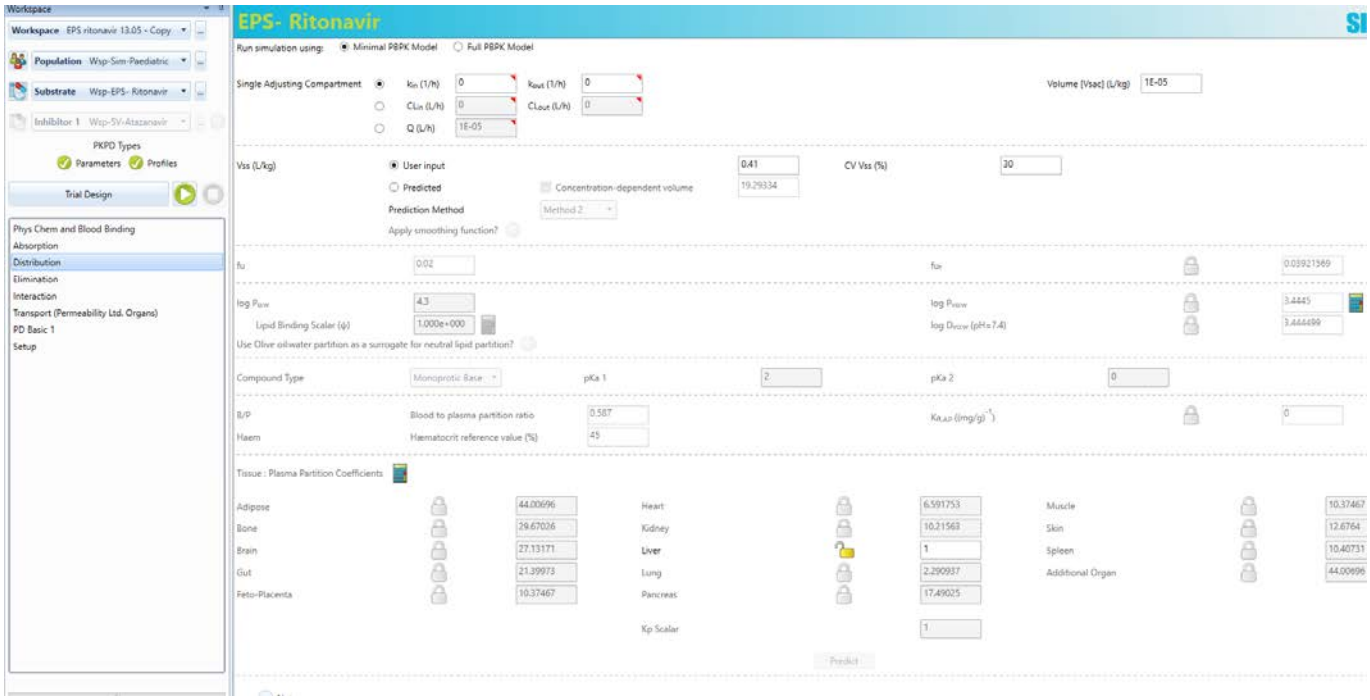


Figure 5 7 Filling the distribution tab with ritonavir details

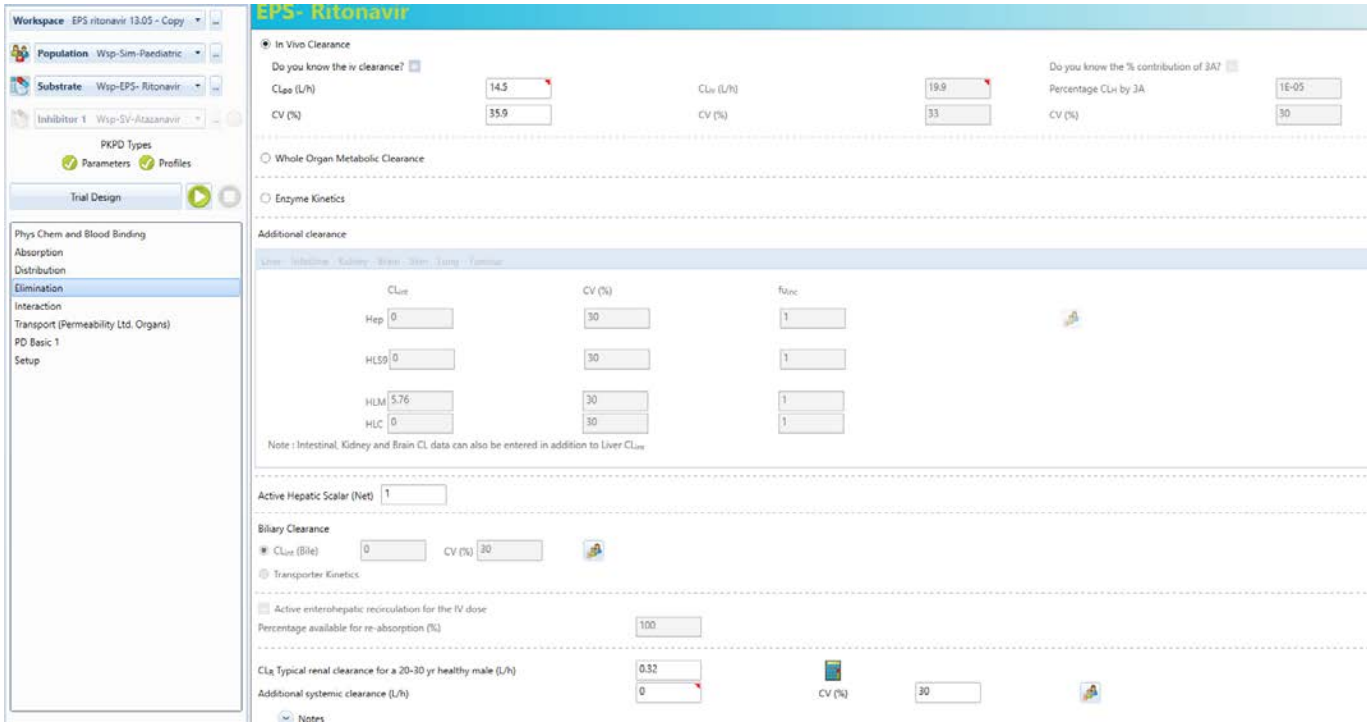


Figure 5 8 Filling elimination tab with ritonavir details

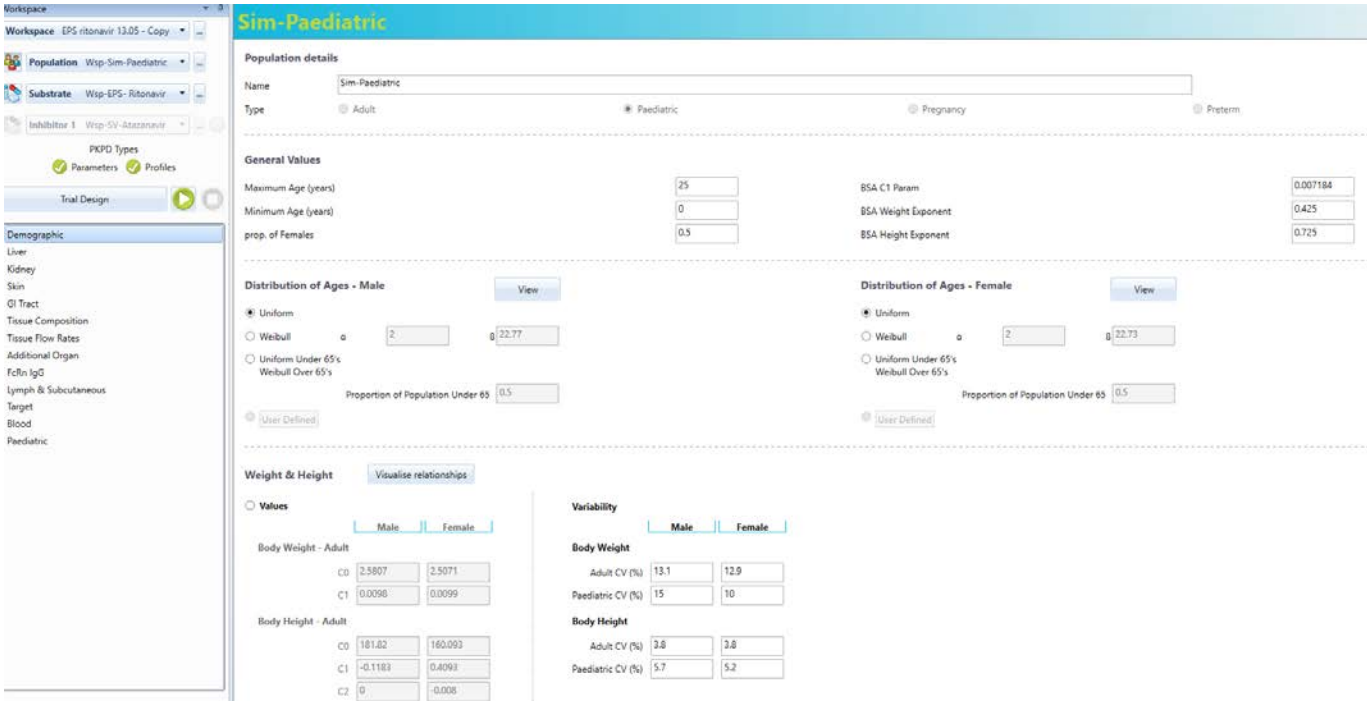


Figure 5 9 Filling the population details at the population tab

The same tab (Population) provides the option of customizing the parameters of the GI tract, which is the ultimate goal of our simulation, in order to update the default mode of the SimCyp paediatric software (Figure 5 9).

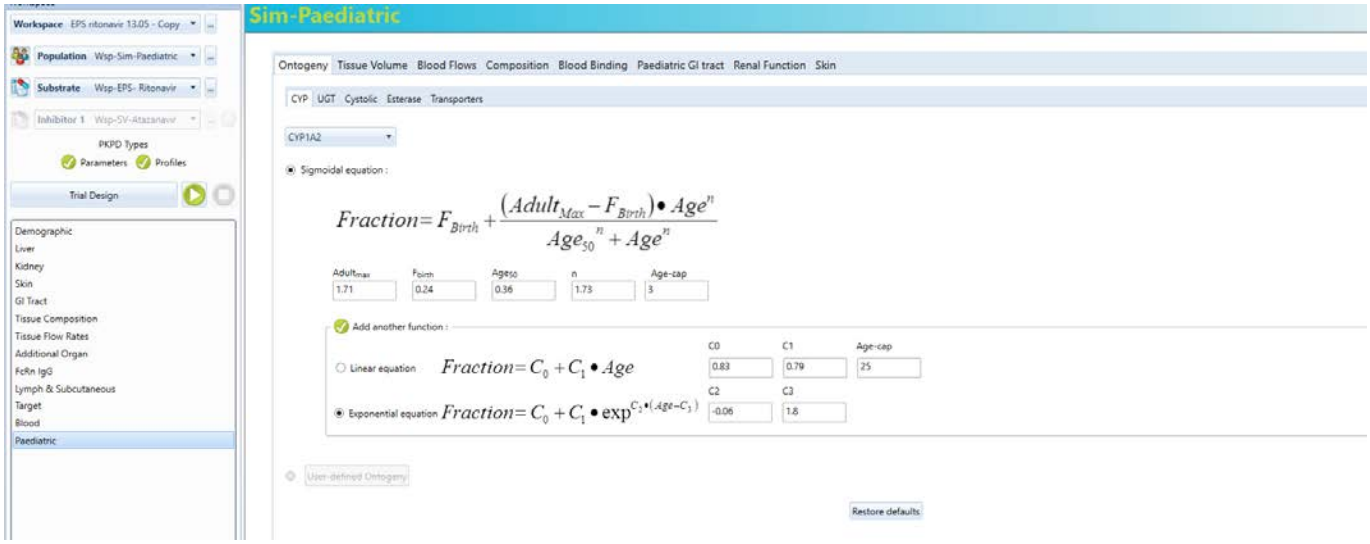


Figure 5 10 Population tab providing the option of customising the GI attributes

The gastrointestinal attributes could be edited accordingly, as seen in Figure 5 10. My study Papadatou Soulou *et al*. [2019] included both fluid fed and fasted patients. It is important that the software provides the option of selecting the feed type, in order to depict the realistic scenario as close as possible. However, as it was expected, the specific feed type (Oral Klean prep solution) and the volume of it could not be input into the parameters.

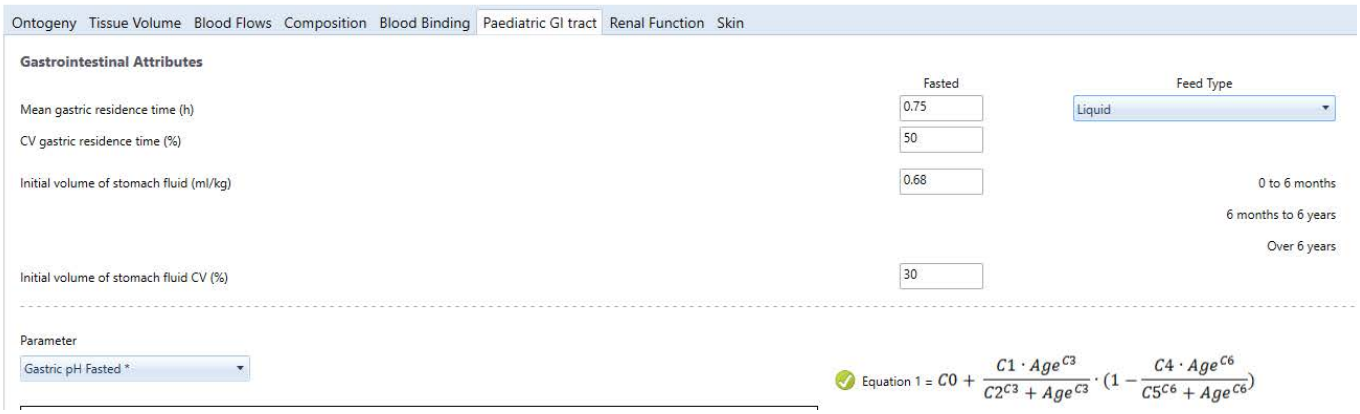


Figure 5 11 SimCyp software provided the option of customising the paediatric GI tract, and selecting the feed type.

It is very important to customise all the aforementioned tabs, otherwise it is possible to be led to under-predictions. In order to start the simulation, the trial button is selected and the trial design is customized, as described in Figure 5 12

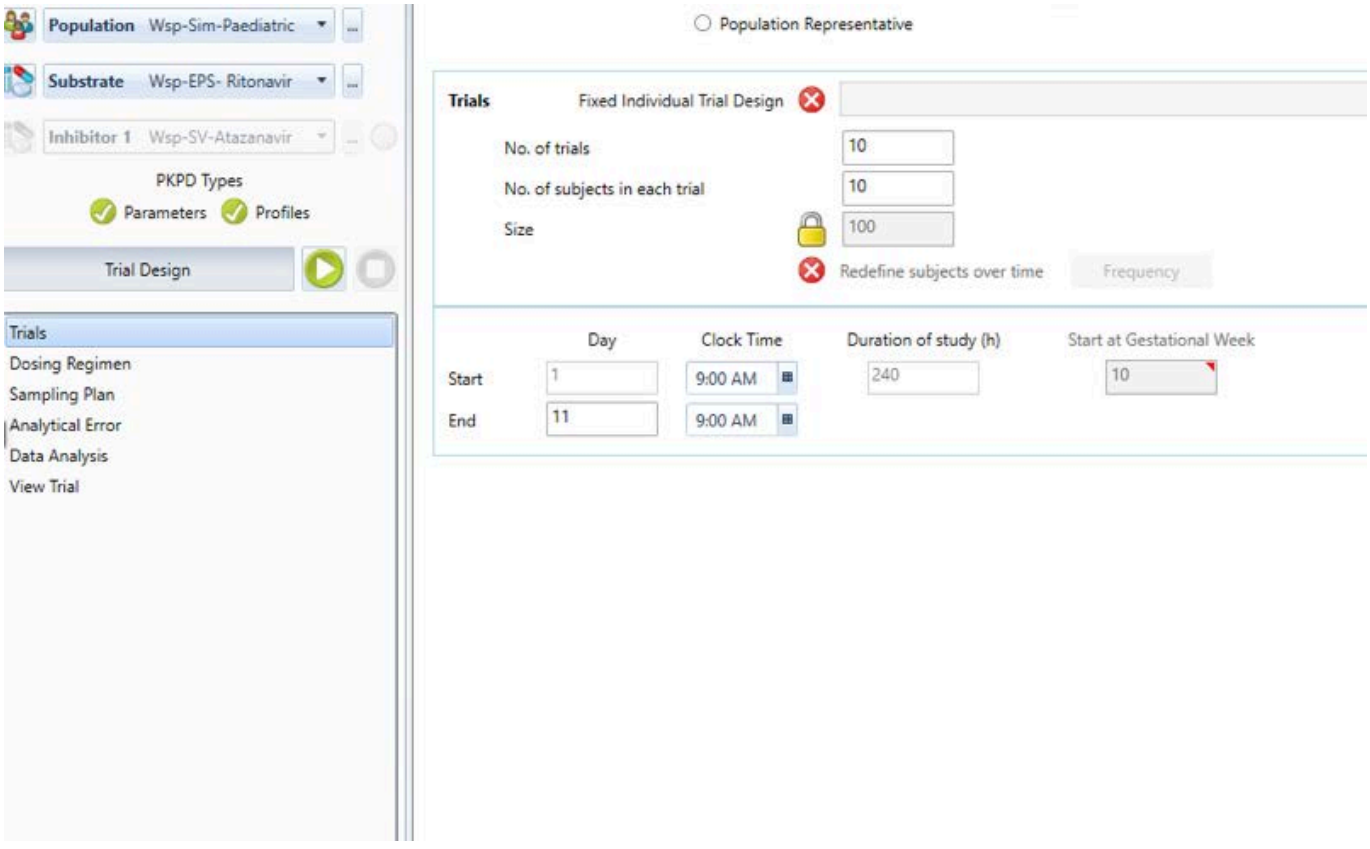


Figure 5 12 Trial design, where the number of trials that will "run" is selected, along with the number of subjects in each trial. It has been decided, that by customising the days of the trial to be 11, it is an adequate amount of days to provide accurate results for the trial.

The excel option has been selected for extracting the data and generating the graphs. Additionally, all the parameters that are of interest to observe are selected as well (Figure 5 13) The Excel file is selected, all the parameters are required to generate graphs and data.

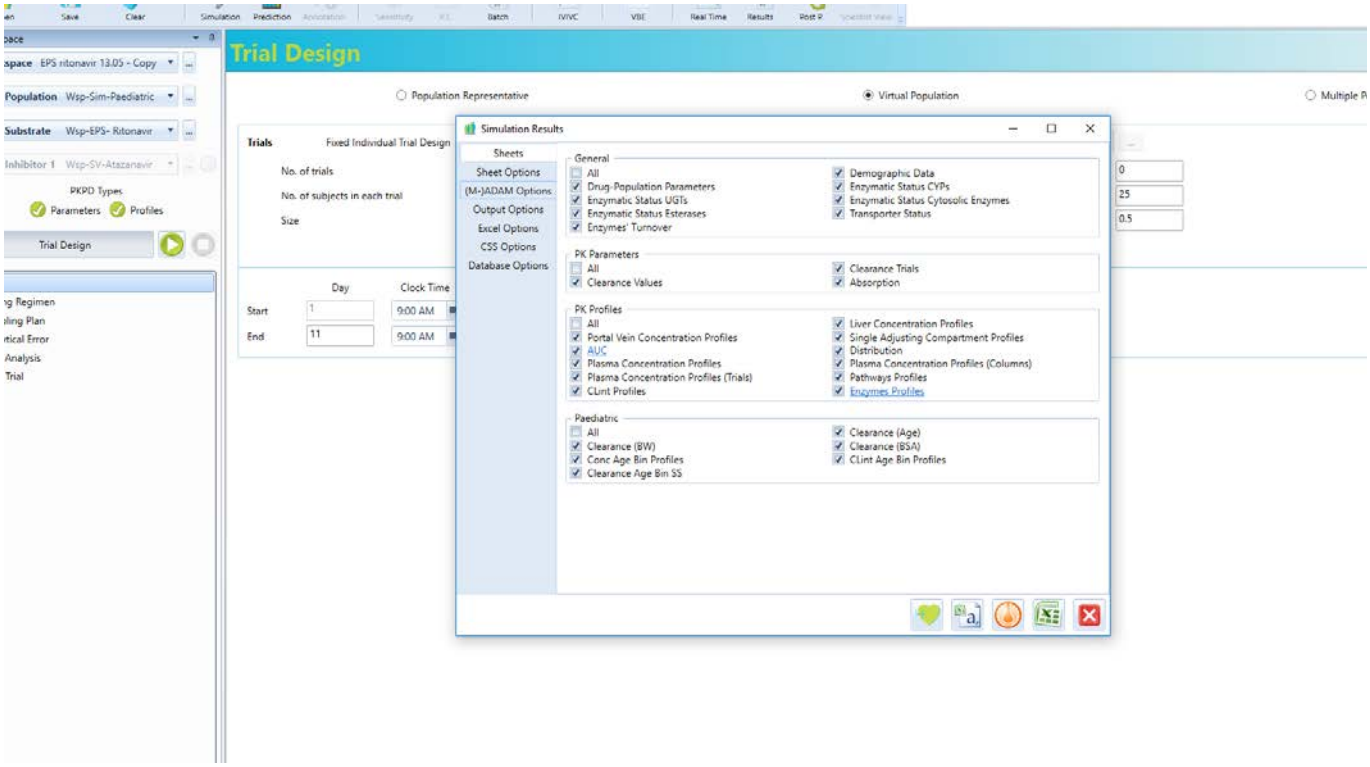
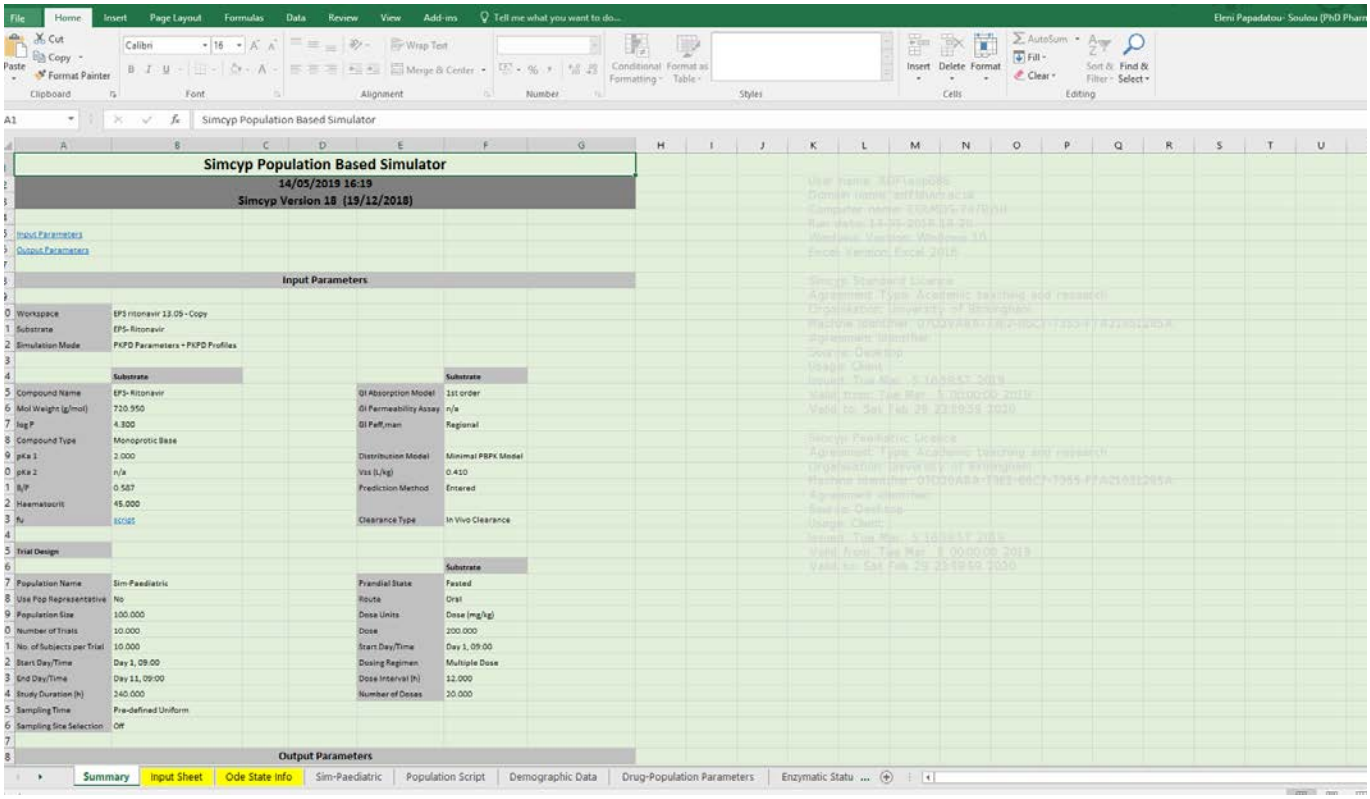


Figure 5 13 Selecting parameters for generating graphs and data for the paediatric simulation study

Important technical detail: neglecting to unselect the "overlay observed data file" could stop the simulation from running.

The simulation needs some time to run. When it is completed, the following excel file is extracted. As seen at the bottom line (yellow highlighted), the file is constructed by multiple spreadsheets, which include a huge amount of information and graphs (Figure 5 14). The researcher's expertise enable the selection of the appropriate graph and information from the simulation data.



Simcyp Population Based Simulator

14/05/2019 16:19

Simcyp Version 18 (19/12/2018)

Input Parameters

Output Parameters

Input Parameters

Workspace	EPS ritonavir 13.09 - Copy			
Substrate	EPS- Ritonavir			
Simulation Mode	PKPD Parameters + PKPD Profiles			
	Substrate		Substrate	
Compound Name	EPS- Ritonavir	GI Absorption Model	1st order	
Mol Weight (g/mol)	720.950	GI Permeability Assay	n/a	
log P	4.300	GI Peff,man	Regional	
Compound Type	Monoprotic Base			
pKa 1	2.000	Distribution Model	Minimal PBPK Model	
pKa 2	n/a	Vss (L/kg)	0.410	
B/P	0.587	Prediction Method	Entered	
Haematocrit	45.000			
fu	script	Clearance Type	In Vivo Clearance	
Trial Design				
			Substrate	
Population Name	Sim-Paediatric	Prandial State	Fasted	
Use Pop Representative	No	Route	Oral	
Population Size	100.000	Dose Units	Dose (mg/kg)	
Number of Trials	10.000	Dose	200.000	
No. of Subjects per Trial	10.000	Start Day/Time	Day 1, 09:00	
Start Day/Time	Day 1, 09:00	Dosing Regimen	Multiple Dose	
End Day/Time	Day 11, 09:00	Dose Interval (h)	12.000	
Study Duration (h)	240.000	Number of Doses	20.000	
Sampling Time	Pre-defined Uniform			
Sampling Site Selection	Off			

Output Parameters

Figure 5 14 Generated excel file (Summary spreadsheet)

6. Chapter 6: Simulated Fluids chapter

Method development by working with simulated intestinal and gastric fluids

6.1 Relevance to thesis

The methodology of the GI chapter (

7. Chapter 7: Gastro-intestinal human samples analysis chapter) was built on the experiments that took place in this chapter. Although using human intestinal fluids has been described as the best medium for drug dissolution experiments [Soderling *et al.*, 2010], their use is limited by ethical restraints and low availability. Simulated intestinal fluids (SIFs) are used instead as the first alternative [Klein, 2010]

6.2 Introduction

The physicochemical properties of the intestinal fluids are crucial for drug dissolution and thus, drug absorption. Fluid pH, osmolality, buffer capacity, bile salt composition and concentration are all important parameters affecting the dissolution process and subsequently, the fraction absorbed. When water or simple buffer is used as a medium for measuring solubility, the result is not always indicative of the solubility in the gastro-intestinal tract [Dressman *et al*., 2007]. It is a recognized that aqueous solubility can lead to an inaccurate prediction of oral bioavailability, the risk of which is greater for poorly soluble and lipophilic drugs [Dressman *et al*., 2007]. Several factors such as gastrointestinal hydrodynamics, transit time and GI media have to be taken into consideration in order to establish biorelevant *in vivo* dissolution methodologies [Soderling *et al*., 2010]. Use of human intestinal fluids (HIFs) to determine *in vivo* solubility and dissolution are described as the "gold standard" [Soderling *et al*., 2010]. However, their use is, for the most part, not feasible due to ethical constraints, limited availability of HIF and variability associated with HIF. Instead, Simulated Intestinal Fluids (SIFs) have been created as an alternative [Klein, 2010]. Efforts have been focused on continuously updating SIFs to ensure that they are biomimetic, as they are used to predict *in vitro* the oral drug absorption of drugs.

FaSSGF (Fasted State Simulated Gastric Fluid), FaSSIF (Fasted State Simulated Intestinal Fluid) and FeSSIF (Fed State Simulated Intestinal Fluid) and FaSSCoF (Fasted State Simulated Colonic Fluid) and FeSSCoF (Fed State Simulated Colonic Fluid) have been created to

represent pre-(fasted) and postprandrial (fed) conditions in the adult gastric, intestinal and colonic environment respectively [Galia *et al*., 1998; Vertzoni *et al*., 2005; Jantratid *et al*., 2008; Vertzoni *et al*., 2010]. Dressman *et al*. [1998] and Galia *et al* [1998] were the first to introduce fasted and fed small intestinal fluid based on adult data. These attempts aimed not only to reflect the pH, buffer capacity and osmolality of adult human intestinal fluids (HIFs), but also to represent bile salts and phospholipids to enhance the bio-relevance of these media. Galia *et al*. [1998] demonstrated that BCS classification of drugs are particularly influenced by these natural surfactants. Later on, Jantrid *et al*. [2008] introduced FaSSIF-V2 and FeSSIF- V2 to better reflect the composition of HIFs in the GI tract and also to achieve a higher stability of the media [Fuchs *et al.*, 2015]. Fuchs *et al*. [2014] identified lysolecithin as the main phospholipid and taurocholate, glycocholate and glycochenodeoxycholate as the main bile salts present in fasted HIF. Furthermore, surface tension and critical micelles concentration (CMC), which is the concentration value above which micelles form, were evaluated and compared with forerunner's media, FaSSIF and FaSSIF-V2, to create the updated version of FaSSIF-V3 [Fuchs *et al.*, 2014]. Table 6 1 presents the composition of the media FaSSIF, FaSSIF- V2 and FaSSIF-V3 that have been developed as adult SIFs. This thesis includes characterisation of SIFs, as they have been used to develop analytical techniques for future use in HIFs.

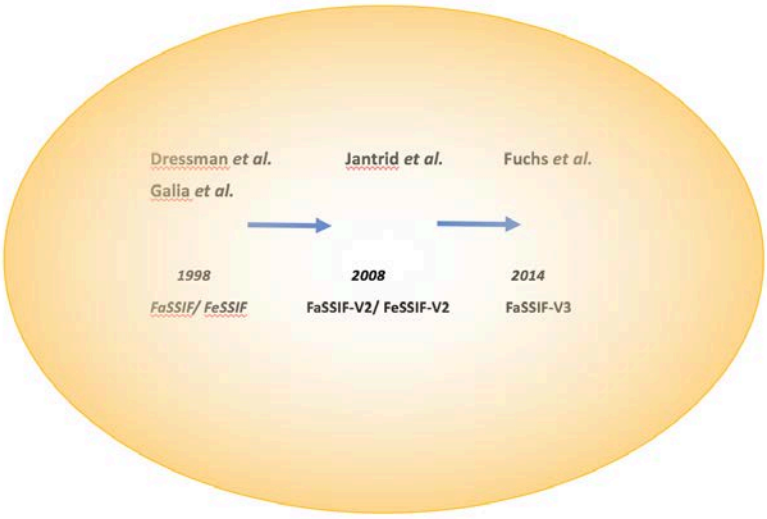


Figure 6 1Figure illustrating the history of development of Simulated Intestinal Fluids. Fuchs *et al.* [2015]

Table 6 1 Composition of media that have been developed as adult SIFs. Fuchs *et al*. [2015]

Medium	FaSSIF	FaSSIF-V2	FASSIF-V3
BS: Taurocholate (mM)	3	3	1.4
PL (lecithin) (mM)	0.75	0.2	0.035
Ratio BS/ PL	4/1	15/1	Approx. 29/1
Buffer	Phosphate	Maleate	Maleate
Osmolality	270	180	215

Several prototypes of FaSSIF-V3 were developed in order to depict the most accurate and close to reality condition. The multiple FaSSIF-V3 were composed by different concentration ratios of different bile salts. Fuchs *et al* [2015] has used taurocholate (TC), glycocholate (GC), tauroursodeoxycholate (TUDC) and glycochenodeoxycholate (GC) have been used, together with the hydrolysis products of lecithin, lysolecithin and sodium oleate**.** It was concluded that FaSSIF- V3- GC/ TC Chol was most able to reflect the best the solubility of the most investigated components in fasted HIF, thus it was referred to in subsequent publications as FaSSIF-V3 [Fuchs *et al*., 2015]. Table 6 2 presents different compositions of several FaSSIF-V3 prototypes.

Table 6 2 Compositions of different FaSSIF-V3 prototypes and final version of FaSSIF-V3-GC/ TC Chol (red highlighted row)
Information from Fuchs *et al*. [2015].

FaSSIF- V3- prototype	Bile Salt(s)	mM	Phospholipids	mM	NaOleate mM	Chol mM
FaSSIF- V3- TC	TC	2.8	PC/ LPC	0.035/0.315	0.315	-
FaSSIF- V3- GC/TC	GC/ TC	1.4/1.4	PC/ LPC	0.035/0.315	0.315	-
FaSSIF- V3- GC	GC	2.8	PC/ LPC	0.035/0.315	0.315	-
FaSSIF-V3- $TC_{\_1/2}$ PL	TC	2.8	PC/ LPC	0.0175/0.1575	0.1575	-
FaSSIF-V3- TUDC	TUDC	2.8	PC/ LPC	0.035/0.315	0.315	-
FaSSIF-V3-TCDC	TCDC	2.8	PC/ LPC	0.035/0.315	0.315	-
FaSSIF- V3- GC/ TC_ Chol	**TC/GC**	**1.4/1.4**	**PC/ LPC**	**0.035/0.315**	**0.315**	**0.2**
FaSSIF-V3- GCDC	GCDC	2.8	PC/ LPC	0.035/0.315	0.315	-

6.3 Aim

The aim of this project was to develop characterization methods on simulated intestinal fluids (SIFs) in order to apply them to the paediatric human intestinal fluid (HIFs) samples.

6.4 Methods

6.4.1 Chemicals:

KH_2OPO_4, HCl, NaCl, NaOH, $NaHPO_4.2H_20$, lecithin, Na- taurocholate, pancreatin, pepsin, deionised water.

6.4.2 Simulated Gastric Fluid (with and without Pepsin)

Simulated Gastric Fluid (with/ without pepsin) was made as described below:

- 2 g of NaCl were added in 0.25 L of deionised water, and dissolved using a magnetic stirrer. Next, 3.20 g pepsin were added in 0.05 L of deionised water and the material was transferred to a sealed container and finally, to the NaCl solution. Following to this, deionised water was added to it until the volume reached 0.9 L. Stirring was required throughout the addition of the water.
  Further on, 0.07 L of HCl 0.01 M was carefully added to the solution. In order to verify that the pH equals to 1.2m the pH should be checked. If required, adjust the pH to this value by addition of HCl. The final solution was transferred to a 1 L volumetric flask.

- The same procedure was repeated for the creation of simulated gastric Fluid without pepsin, except for the step of pepsin addition. It can be stored for 7 days in absence of pepsin and for 3 days in the presence of pepsin.

6.4.3 Simulated Intestinal Fluid (with and without Pancreatin)

Simulated Intestinal Fluid (with/ without pancreatin) was made as described below:

- A solution of 0.2 M NaOH was created by adding 4 g of the substance to 0.25 L deionised water to a 0.5 L volumetric flask and making up to volume with deionised water. The procedure was carried out within a fume cupboard. Then, 6.7 g of potassium dihydrogen phosphate and 0.077 L NaOH was added to 0.5 L deionised water. The solution was dissolved using a magnetic stirrer. Next, 10 g of pancreatin was weighed within an empty container that could be sealed. A small volume of deionised water (about 0.05 L) was added to it, so as to reduce the risk of exposure. The volume was made up to about 0.9 L with deionised water in a large beaker, while stirring gently.

The pH was checked and found to be 6.8. Otherwise, it was adjusted to this value with NaOH or HCl. The final solution was transferred to a 1 L volumetric flask and made up to the 1 L mark with deionised water.

- The same procedure was repeated for the creation of simulated intestinal fluid without pancreatin, except for the step of pancreatin addition. It can be stored for 7 days in absence of pancreatin and for 3 days in its presence.

6.4.4. Blank FaSSIF

Fasted state simulated intestinal fluid was made as described below:

- About 0.65 L deionised H_20 was used and 0.348 g Sodium Hydroxide pellets, together with 4.470 g $NaHPO_4.2H_20$ and 6.186 g NaCl, were added into it. The solution was dissolved, using a magnetic stirrer. Next, the volume of the solution was made up to 0.9 L with deionised H_20, while continuously stirring. The pH should be checked and found to be 6.5. Further on, the solution was transferred to a 1L volumetric flask and made up to the 1L mark with deionised H_20. Blank FaSSIF is stable for seven days, then it should be disposed.

6.4.5 Fasted State Simulated Intestinal Fluid (FaSSIF)

- For the composition of FaSSIF, 1.65 g of sodium taurocholate and 0.59 g of lecithin were added to 0.2 L of blank FaSSIF. The solution was stirred overnight on a magnetic stirrer to create a clear micellar solution. The next day, the micellar solution was transferred to a 1 L volumetric solution and the volume was made up with blank FaSSIF until the top.

  Final solution of FaSSIF contains 3 mmol/L sodium taurocholate (NaTC), 0.75 mmol/L lecithin, pH 6.50 and has an osmolality of 270 mOsm.

6.4.6 Blank FeSSIF

Fed state simulated intestinal fluid was made as described below:

- Approximately 0.5 L H_20 was used, and 4.04 g NaOH, 144 ml of 1M acetic acid and 11.874 g NaCl were added to it. The solution was stirred using a magnetic stirrer. Then, deionised H_20 was added until the volume of the solution was 900 ml, while stirring

continuously. The pH of the solution should be checked and found to be 5. Further on, the solution was transferred to a 1 L volumetric flask and made up with deionised H_20. Blank FeSSIF can be stored for seven days

6.4.7 Fed State Simulated Intestinal Fluid (FeSSIF)

- 8.25 g of Sodium Taurocholate, together with 2.95 g of lecithin, were added to 0.2 L blank FeSSIF, and stirred overnight on a magnetic stirrer. Next day, the micellar solution was transferred to a 1 L volumetric flask and made up to 1 L volume with blank FeSSIF. FeSSIF was composed by 15 mmol/L sodium taurocholate (NaTC), 3.75 mmol/L lecithin, with a pH value of 5.00 and an osmolality of 670 mOsm.

6.4.8 Viscosity:

In order to determine the viscosity of the SIFs, Lovis viscometer 2000 was used. Prior to the initiation of the measurements, Lovis density was set as equal to the density of water (0.9882 mg/ cm^3) and a check with water sample was conducted via filling the capillary with 0.001 L of sample. After the successful completion of the water check, 1 ml of gastric fluid with pepsin was injected in the capillary to obtain the viscosity value. The same procedure was repeated for the rest of the fluids. Before starting a new measurement, it was essential to clean extensively the remaining of the previous sample with water and air pump. The results demonstrated both dynamic and kinetic viscosity values. However, only dynamic viscosity (mPa/s) was considered in terms of comparing the viscosity values of the simulated fluids and kinetic values were recorded for consideration.

6.4.9. pH:

pH measurement was conducted using a Hanna HI 2210 pH meter. The actual pH values of the simulated fluids were recorded on the day of their preparation. Minor deviations (0.03-0.2) from the hypothetical value were acceptable. Hanna HI 2210 pH meter probe was placed within a solution of HI7071 (3.5 M KCl + AgCl electrolyte solution) when not in use. Prior to the recording of the values, calibration of the pH meter was required. Furthermore, the temperature must be set at the actual temperature of the measured solutions, as it can influence the accuracy of the pH measurements. After the pH reading was stabilised, the value was recorded as the actual pH value. When each pH recording was completed, the probe was

carefully rinsed with water. The next day the pH values were repeated and recorded for all the simulated fluids.

6.4.10 Osmolality (mOsm/kg):

The measurement of osmolality via Osmomat 3000 is based on the principle of freezing point depression. 50 ul of Fluid was accurately measured using a 20-200 ul Thermoscientific pipette into a vessel. Then, the thermistor probe was manually placed into the vessel and the measurement started automatically. After recording the osmolality value that was displayed, the thermistor should be gently cleaned with a clean towel. The same procedure was repeated for each individual simulated fluid and the osmolality values were recorded.

6.4.11 Buffer Capacity:

Buffer capacity measurement took place by using a calibrated Hanna HI 2210 pH meter.
Buffer capacity is defined as the capacity of the solution to resist to 1 unit change of pH. According to the literature, either 1M HCL or 1M NaOH are used for titrating and thus, calculating the required quantity of needed volume. Buffer capacity is calculated by the formula:

Equation 6 1

$$\beta = \Delta AB / \Delta pH$$

Where: AB= the amount of acid 1M HCL or base 1M NaOH added to alter the pH 1 unit.

50 ml of sample were titrated with 1 M HCL. The procedure of volume measurement took place via pipetting 50 µl each time, and recording the required amount of acid to change pH by one unit. Knowing the volume that induced a specific ΔpH, buffer capacity could be calculated via Equation 6 1. The process took place following the protocol of Perez M *et al*. [2006]. The same procedure was repeated for all the simulated fluids.

6.5 Results

6.5.1 Viscosity

Figure 6 2 demonstrates the viscosity values measured at the simulated fluids' analysis that took place in UoB laboratory settings. FeSSIF was characterised by the highest viscosity of all simulated fluids, with the value of 1.0903 mPa/s. Then, FaSSIF and intestinal fluid with pancreatin followed, exhibiting a slight decrease compared to the viscosity of FeSSIF. Next, in] descending order in terms of viscosity was gastric fluid with pepsin, while gastric fluid

without pepsin and intestinal fluid without pancreatin were the less viscous of all simulated fluids.

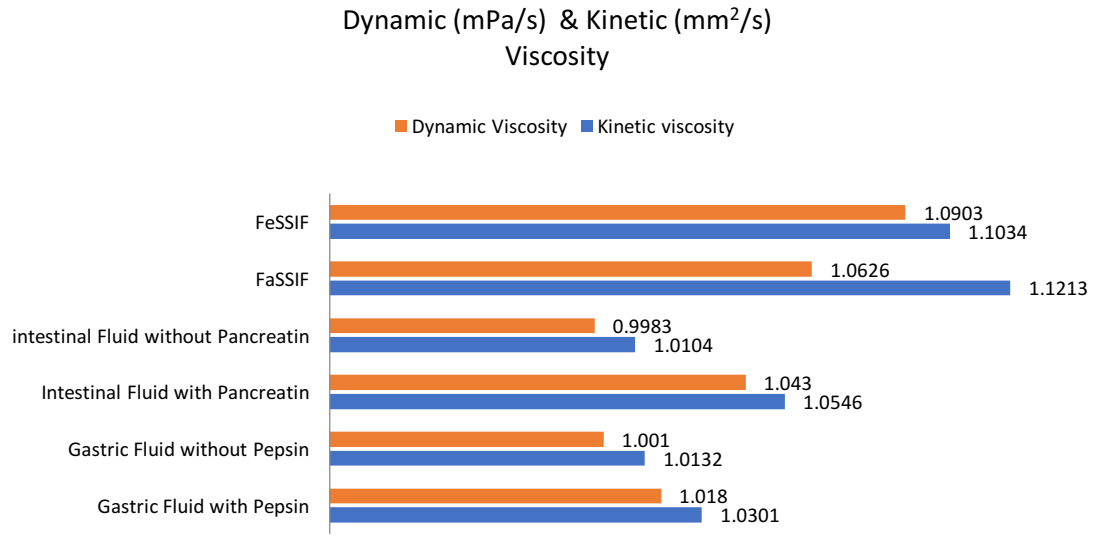


Figure 6 2 Mean viscosity values of the simulated fluids: ***gastric fluid with and without pepsin, intestinal fluid with and without pancreatin, FaSSIF and FeSSIF.***

6.5.2 pH

Figure 6 3 demonstrates the pH values measured at the simulated fluids' analysis that took place in UoB laboratory settings. Both gastric fluids with pepsin and without pepsin were highly acidic on both days no.1 and no.2 (day of preparation and next day). On the other hand, FaSSIF and FeSSIF were close to neutral 7(6.48 and 6.78 respectively). Intestinal fluids with and without pancreatin were characterised as neutral, as they exhibited the highest p (6.93 and 6.78).

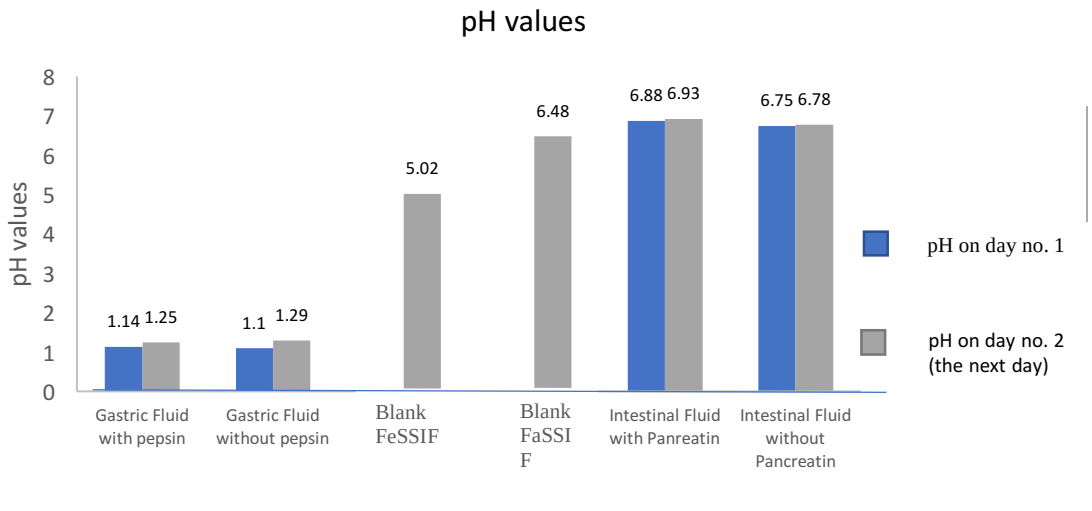


Figure 6 3: pH values as recorded on days no.1 and no.2. FeSSIF and FaSSIF are characterised only by one column as they have been prepared straightout on day no.2 (blank FaSSIF and blank FeSSIF pH is the same prior and post addition of bile salts)

6.5.3 Osmolality

Figure 6 4 demonstrates the osmolality values measured at the simulated fluids' analysis that took place in UoB laboratory settings. The highest osmolality value was recorded for FeSSIF (636 m0sm/kg). Then, FaSSIF was the second one in descending order in terms of osmolality. Further on, it is observed that Gastric fluids with and without pepsin present similar osmolality numbers (219 and 200). The same is observed for intestinal fluids with and without pancreatin (97 and 105), which have the lowest osmolality values of all simulated fluids.

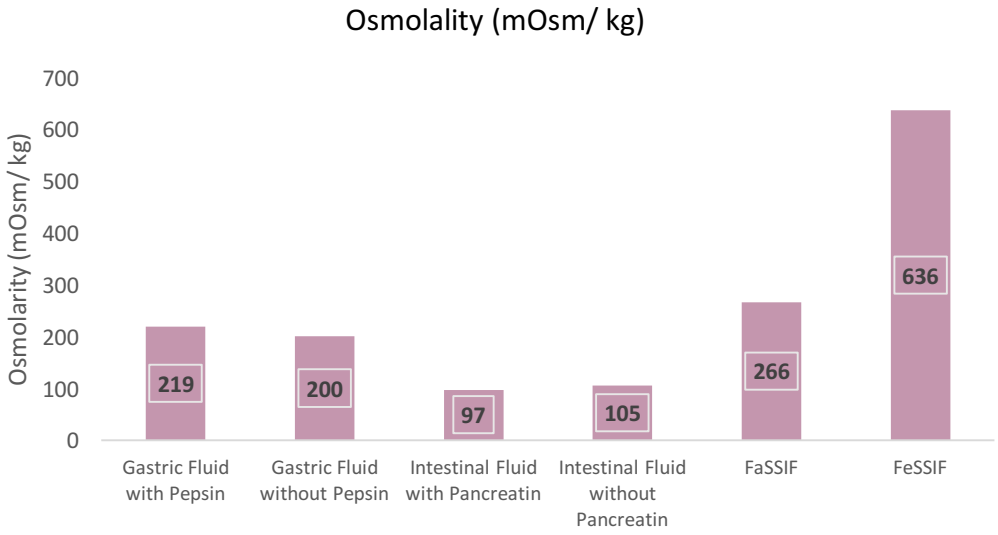


Figure 6 4: Osmolality values of the simulated fluids: *gastric fluid with and without pepsin, intestinal fluid with and without pancreatin, FaSSIF and FeSSIF*.

6.5.4 Buffer Capacity

It is observed that Gastric fluids (with and without pepsin) have the greatest buffer capacity values from all the simulated fluids under scrutiny, with values of 5 and 4.16 respectively. The buffer capacity of FeSSIF was 2.63, demonstrating a 47.4% decrease of the highest buffer capacity. FaSSIF, intestinal fluids with and without pancreatin were characterised by similar low buffer capacity values (0.31, 0.405 and 0.28).

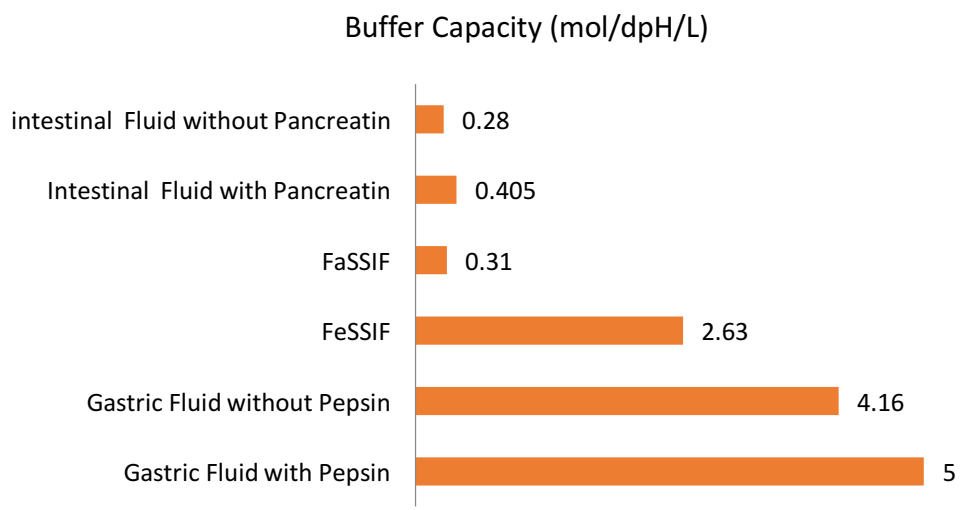


Figure 6 5 Buffer capacity values of the Simulated Fluids *gastric fluid with and without pepsin, intestinal fluid with and without pancreatin, FaSSIF and FeSSIF*.

6.6 Discussion

When measuring the viscosities of the simulated fluids, several challenges were encountered. More viscous fluids, such as the intestinal fluid with pancreatin, were reported as "unstable" and the instrument could not provide specific values. To overcome this, the injected sample with e.g pancreatin was passed through a mini- filter, that was attached to the capillary. When doing so, the lovis viscometer seemed to be able to detect a measurable viscosity value. However, the limitation that only solutions could be measured meant that it would not be possible to characterise the human intestinal samples as they are not simple solutions and will contain solid particulate materials that will prevent measurement

Regarding buffer capacity, the volume of the simulated fluid that was used for determining each value was 50 ml. However, when the actual human samples volume is considerably lower with volumes ranging from 1 to 5 ml maximum. pH measurements took place on the day of preparation and the next day. Between the values obtained on day 1 and day 2, minor deviations were observed. However, these deviations fall within acceptable range (0.03- 0.2) and were considered insignificant.

As SIFs characterisation was carried out based on protocols, which are widely available for the creation of FaSSIF and FeSSIF versions, it was expected that the measured values should be ideally identical with the theoretical values of the SIFs. Table 6 3 provides a summary of how our values fit with the SIF literature data. It is obvious that our values for FaSSIF-UoB and FeSSIF-UoB compare directly to the FaSSIF and FeSSIF biorelevant media when it comes to their pH and osmolality values. FaSSIF-V2, FeSSIF-V2 and FaSSIF-V3 compositions are also

listed in Table 6 3 to be used as reference values. It is observed that their values may not be identical with our values regarding pH and osmolality, but they are very similar with them (FaSSIF-UoB & FeSSIF-UoB). Viscosity values are not included in the table below, as HIFs will not be characterised in

7. Chapter 7: Gastro-intestinal human samples analysis chapter in terms of viscosity. Regarding buffer capacity, values for $FaSSIF_{UOB}$ and $FeSSIF_{UOB}$ were 0.31 and 2.61 mol/L/DpH, while buffer capacity as reported in the literature for FaSSIF, FeSSIF, FaSSIF-v2, FeSSIF-V3 is 0.012, 0.072, 0.01, 0.025 and 0.0056 mol/L/dpH. [Klein, 2010; Mudie *et al.*, 2020]. This variation could be attributed to two reasons; first, due to the large volume (50 ml) that was used for determining buffer capacity. When the same experimental process was repeated for HIFs, a considerably lower volume (up to 5 ml) was used and buffer capacity was easier to determine this way. Secondly, buffer capacity was recorded last of the rest properties (pH, osmolality & viscosity); pH could drift over time, affecting buffer capacity as well.

Table 6 3 A comparison between the SIF values as they have been measured in our lab (UoB) and the literature reference values of FaSSIF, FeSSIF, FaSSIF-V2, FeSSIF-V2 and FaSSIF- V3.

	FaSSIF-UoB	**FeSSIF-UoB**	**FaSSIF** [Galia *et al.*, 1998; Dressman *et al.*, 1998]	**FeSSIF** [Galia *et al.*, 1998; Dressman *et al.*, 1998]	**FaSSIF-V2** [Jantrid *et al.*, 2008]	**FeSSIF-V2** [Jantrid *et al.*, 2008]	**FaSSIF-V3** [Fuchs *et al.*, 2015]
pH	6.48	5.02	6.5	5	6.5	5.8	6.7
Osmolality (mOsm/Kg)	266	636	270	635	180	390	215

As seen from Table 6 3, the developed laboratory methods are repeatable and can provide accurate and consistent results. The recorded values were in accordance with literature values, and therefore, these methods will be used in human intestinal fluids' characterisation in

7. Chapter 7: Gastro-intestinal human samples analysis chapter.

7. Chapter 7: Gastro-intestinal human samples analysis chapter

Characterisation of fasted state gastric and intestinal fluids collected from children

7.1 Relevance to thesis

Exploring the physicochemical characteristics of paediatric human intestinal fluids (HIFs) will enable a deeper understanding of their impact on oral drug absorption. At the moment, there is conflicting or missing information for the majority of these parameters, especially for paediatrics. Ethical constraints have limited paediatric research in this field so far. My work has overcome these obstacles as this research was a retrospective study. I have collected GI samples from children undergoing endoscopy as a part of their clinical routine scan. Neither invasive processes have taken place nor alterations have been conducted in their clinical protocol. Until now, conflicting information exists for paediatric pH values and limited knowledge is available for other physicochemical values, as buffer capacity, osmolality, and bile salts' composition of the paediatric HIFs. My project aimed to fill these gaps to update the current *in vitro* methods for predicting paediatric oral drug absorption. The research was funded by Janssen pharmaceuticals, and we collaborated closely with Greisfwald University in Germany. They will use our generated values to update the currently used *in vitro* tools. This work is of great significance as by updating the *in vitro* tools with updated values, we will be able to predict as realistically as possible the challenging paediatric oral drug absorption.
A major project within this thesis was the collection and analysis of paediatric gastric and intestinal fluids. The work conducted has been written as a publication that is presented in the following chapter.

7.2 Synopsis

Study Title	Characterisation of fluids and mucosal tissues from paediatric stomach and small intestinal tract to enable development of biorelevant models to predict drug absorption
Internal ref. no. / short title	PaedGIFT (**Paed**iatric **G**astro-**I**ntestinal **F**luid and **T**issue)
Study Design	Experimental, prospective study with non-probability sampling

Study Participants	Children undergoing endoscopy as part of their clinical care. The children are stratified using the WHO criteria: *Neonate: 0-30 days* *Infant: 1 month-2 years* *Young child: 2-6 years* *Child: 6-12 years* *Adolescent: 12-15 years*
Planned Sample Size	Similar studies use up to 20 participants for characterisation. We planned to obtain 20 samples from each of the following populations *Neonate: 0-30 days* *Infant: 1 month-2 years* *Young child: 2-6 years* *Child: 6-12 years* *Adolescent: 12-15 years* Endoscopy is typically performed in children >30 days old so a total of 80 samples from endoscopy were anticipated. (20 for each age group from infant to adolescent)

Table 7 1 PaedGIFT Study Summary

7.3 Aim of the Study

The aim of the study was to characterise paediatric gastrointestinal fluids with regard to their pH, buffer capacity, osmolality, and bile salts.

7.4 Objectives of the Study and Outcome Measures

Table 7 2 PaedGIFT Study's objectives and outcome measures

Objectives	Outcome Measures
Primary Objective: To characterise the properties of gastric and small intestinal fluids from children in terms of: pH, buffer capacity, osmolality, and the qualitative and quantitative composition of bile salts	Measurements of the pH, buffer capacity, osmolality, and the qualitative and quantitative composition of bile salts from collected fluids.

7.5 Study design

Ethical approval was granted by South Birmingham NRES Committee (IRAS 251909).
This study was an experimental, prospective study with non-probability sampling.

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Title: Characterisation of fasted state gastric and intestinal fluids collected from children

Authors: Pawar. G., Papadatou-Soulou. E., Mason, J., Muhammed, R., Watson, A., Catherine, C., Abou-Elwafa Abdallah, M., Harrad, S., Mackie, C., Arien, T., Inghelbrecht, S. and Batchelor, H.

Researcher's contribution to the study: My contribution to the study was writing the ethical applications. Additionally, I was responsible for safety storage and handling of samples on UoB premises. I have undertaken special training to be qualified as a researcher for this. My training included:

- *Good Clinical Practice: 08.06.2017 (provided by the NHS)*
- *Communication and Consent within the paediatric research setting: 13.06.2017 (provided by the NHS)*
- *Research Data and confidentiality: 13.04.2017 [(provided by the medical research council (MRC)]*
- *Good Research Practice: 10.04.2017 [(provided by the medical research council (MRC)]*
- *Research and Human tissue legislation 10.04.2017 [(provided by the medical research council (MRC)]*

Statement: *"The author made a substantial contribution to the conception and design of the study, to the organisation of the conduct of the study, to carrying out the study and to analysis and interpretation of study data. Additionally, the author helped draft the output and critique the output for important intellectual content".*

I have collected the samples when coming to the university's facilities and characterised the collected fluids with regard to their pH, buffer capacity and osmolality. The characterisation methods were developed primarily on simulated intestinal fluids (SIFs) and then were applied on the collected HIFs. I also created the relevant graphs/ figures and analysed the generated data. Bile salt measurements was done by Gopal Pawar, who characterised quantitatively and qualitatively. Research Gopal Pawar developed the bile salts identification and quantification method and analysed the corresponding figures/ tables.

Julie Mason has proofread the article and this chapter, Rafeeq Muhammed was the responsible doctor during the endoscopy process, Alison Watson and Catherine Cotter were the research nurses who gathered and sent the samples to the university facilities. Mohamed Abou- Elwafa Abdalah and Stuart Harrad have supported the bile salts project, while Claire Mackie, Tina Arien and Sabine Inghelbrecht are the Janssen's team that funded the project. Hannah Batchelor has been the PI of the project and my supervisor during the PhD.

Researcher's (EPS) contribution to the article: My contribution to the article writing process was collecting the literature for pH, buffer capacity and osmolality existing values. I have

written the introduction part which corresponds to the need to update the pH, buffer capacity and osmolality values, the materials and methods section for the physicochemical values characterisation. Additionally, I have written the results and discussion part that refers to the physicochemical values of HIFs and generated the corresponding tables and graphs. Researcher's Gopal Pawar work has been on bile salts, in introduction, methods, results and discussion. He also generated the corresponding graphs and tables.

7.6 Abstract

Fundamental knowledge about the composition of intestinal fluids in paediatric populations is currently unavailable. This study aimed to characterise gastric and intestinal fluid from paediatric populations.

Gastric and intestinal fluid samples were obtained during routine clinical endoscopy from paediatric patients at a large teaching hospital. These fluids were characterised to measure the pH; buffer capacity; osmolality; bile acid concentration and composition.

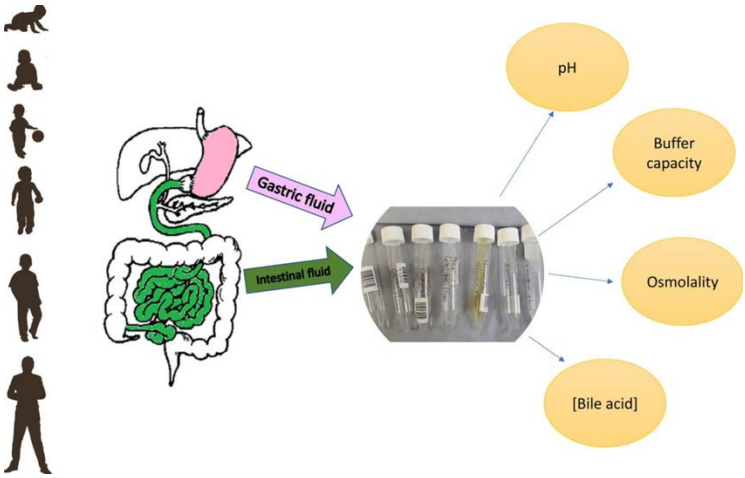
A total of 55 children were recruited to the study aged from 11 months to 15 years of age where 53 gastric fluid samples and 40 intestinal fluid samples were obtained. pH values recorded ranged from pH 0.57 to 11.05 (median: 2.50) in gastric fluids and from 0.89 to 8.97 (median: 3.27) in intestinal fluids. The buffer capacity did not change significantly between gastric and intestinal fluids with median values of 12 mM/L/ΔpH for both fluids. Gastric fluid osmolality values ranged from 1 to 615 mOsm/kg, while intestinal fluid values ranged from 35 to 631 mOsm/kg.

Gastric fluid bile acid concentrations ranged from 0.002 to 2.3mM with a median value of 0.017 mM, whilst intestinal fluid bile acid concentrations ranged from 0.0008 to 3.3mM with a median value of 0.178mM. Glycocholate; taurocholic acid; glycochenodeoxycholate and taurochenodeoxycholate were the most commonly identified bile acids within paediatric intestinal fluids.

All compositional components were associated with large inter-individual variability. Further work is required to develop simulated paediatric media and to explore the impact of these media on drug solubility and dissolution.

Keywords: gastrointestinal fluid; paediatric; bile acid; buffer capacity; pH; osmolality

7.7 Graphical abstract



7.8 Introduction

Drug solubility within the gastrointestinal (GI) tract is key to oral biopharmaceutics parameters including calculation of the maximum absorbable dose [Sun *et al.,* 2004; Lindahl *et al*., 1997] and biopharmaceutics classification system [Amidon *et al*., 1995]. Inadequate solubility can limit absorption of certain active pharmaceutical ingredients (APIs) so it is important to accurately measure solubility in GI fluids. However, GI fluid is a complex media known to exhibit high inter-individual variability. Critical to the prediction of the oral absorption of drugs in children is knowledge of the physical environment within the paediatric intestinal tract. Fundamental knowledge about the composition of intestinal fluids in neonates and children is currently unavailable.

There are several studies where GI fluids have been collected and characterised in the biopharmaceutics arena. The majority of studies have been conducted on adult populations in the fasted state [Lindahl *et al*., 1997; Pedersen *et al*., 2000; Persson *et a*l., 2005; Clarysse *et al*., 2009; Heikkila *et al.*, 2011; Perez *et al*., 2006; Foltz *et al*., 2015; Holmstock *et al*., 2013; Riethorst *et al*., 2016] yet there are also studies exploring the fed state [Pentafragka *et al*., 2019; Vertzoni *et al.,* 2012]. Previous studies conducted in adults and children [Van den Abeele *et al.,* 2018], where fasted fluid was collected and characterised, are listed in Table 7 3. Methodology associated with the measurement of GI fluid have varied and there has been recent work published to standardise methods of assessment [Litou *et al*., 2020].

The development of simulated adult intestinal fluids based on aspirated intestinal fluids has shown superiority in predicting *in vivo* performance compared to simple buffers [Dressman *et a*l., 2000]. Currently used simulated intestinal fluids: fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) are based on adult data sets [Kleberg *et al*., 2010]. However, it is recognised that the GI environment in children may be different to that in adults [Batchelor *et al.,* 2014]. There have been reports that the differences in volumes of fluid present may affect the classification of APIs in children according to the adult biopharmaceutics classification system [Batchelor *et al.,* 2016; del Moral Sanchez *et al*., 2018; Delmoral- Sanchez *et al*., 2019; Bhatt- Mehta *et al.,* 2020l Papadatou Soulou *et al*., 2019].

A comprehensive review [Maharaj *et al*., 2016] on paediatric GI fluids and the component materials revealed several differences in paediatric fluids compared to adult data. The findings included a relatively higher gastric osmolality (of 253 mOsm/L in infants at 8 months) compared to values reported in adults, no reports of bile concentrations or buffer capacity

from paediatric intestinal fluids were found in this review. Based on this review, recipes for paediatric fasted state simulated gastric and intestinal fluids were proposed for both neonates and infants, these reflected worse case scenarios rather than informed compositional content [Maharaj *et al*., 2016]. Subsequent to the Maharaj *et al* (2016) review [Maharaj *et al*., 2016], a study investigating the composition of gastric fluid in a paediatric population was published [Van den Abeele *et al.,*2018]. This gastric fluid study reported pH values ranging from 1.2-8.3 in neonates up to 20 days old (a similar pH range is observed for infants although details are not listed); 0.93-8.15 in children (2-12 years) and 1.24-6.96 in adolescents (12-18 years). The majority of the osmolality values measured in gastric fluids were between 200-350 $mOsmKg^{-1}$ for neonates and infants, with lower mean values of 152 ±74 mOsmKg-1 in children and 196 ±73 mOsmKg-1 in adolescents. Bile salt concentrations in gastric fluids were also measured and large variability was shown: for neonates, the concentration ranged from 0 to 5.6 mM (mean 0.19mM); infants ranged from 0-1.6 mM (mean 0.24 mM); children 0-1.1 mM (mean 0.10 mM) and for adolescents ranged from 0-6.3 mM (mean 0.76 mM) [Van den Abeele et al., 2018].

Bile acids are chemically similar compounds based on a steroid nucleus, when these acids are conjugated to sodium they are termed bile salts and often the terms bile acid and bile salt are used interchangeably. Differences in the number and position of hydroxyl groups in relation to the steroid structure dictate the specific bile acid present. The structure informs the balance between hydrophobic and hydrophilic components within the bile acid which in turn affects how the bile interacts with other chemicals, including how this may affect the solubility of an API [Mithani *et al*., 1996]. Thus, knowledge of bile acid composition within the intestinal fluid is critical and has previously been shown to have very large effects on API solubility [Madsen *et al*., 2018; Perrier *et al*., 2018]. Primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDC) are produced by the liver and conjugated to the amino acids glycine or taurine [Hofmann *et al.,* 1999].

Several studies have reported the bile acid concentration in adult human intestinal fluids, a review of this literature suggested an overall mean bile acid concentration in the fasted duodenum of 3.3 mM and 3.0 mM in the fasted jejunum [Fuchs and Dressman, 2014]. With regard to bile acid composition, adult studies have shown discrepancies in the bile acids detected and all data shows large inter subject variability that could explain these differences [Perez de la Cruz *et al.,* 2006; Riethorst *et al.,* 2016; Reppas *et al.,* 2015]. There is limited data

available on bile acids from paediatric populations. Studies reporting bile acid concentrations in the GI fluids of children are listed in Table 7 3. One study [Van den Abeele *et al*., 2018] reported relative bile acid compositions of gastric fluid with slight differences found between neonate and infant populations to that of children and adolescents, further details are provided in Table 7 3.Drug solubility in the intestine drives absorption for certain APIs and small changes in solubility can have large effects on the absorbed dose and therefore subsequent therapy. The composition of GI fluid, therefore, influences drug product performance and may differ between children and adults. In the paediatric population, knowledge of GI fluid composition is essential to develop and build bio-relevant physical and in silico models with the potential to minimise the burden of clinical trials in children. This study seeks to characterise gastric and intestinal fluid from paediatric populations to include reports on bile acid concentration and composition and to compare these fluids to data from adult populations as well as gastric data from paediatric populations [Van den Abeele *et al.,* 2018].

Table 7 3 Summary of cohort details from studies reported where bile acid concentrations were measured in paediatric population. Glycocholic acid (GC); glycochenodeoxycholic acid (GCDC); glycodeoxycholic acid (GDC); glycoursodeoxycholic acid (GUDC); taurocholic acid (TC); taurochenodeoxycholic acid (TCDC); taurodeoxycholic acid (TDC); tauroursodeoxycholic acid (TUDC); taurolithocholic acid (TLC); deoxycholic acid (DC), lithocholic acid (LC), and ursodeoxycholic acid (UDC).

Reference	Population age	Fed/Fasted	Fluid	Bile acid/salt concentration	Bile acids present
[Norman *et al.,* 1972]	8 healthy neonates (3-15 days)	4 hours after last meal	Duodenal	0.50-5.29 mM (fasting values) Mean value = 2.09 mM	Taurine and glycine conjugates c cholic and CDC acids (TC; TCDC GC; GCDC). The total concentrations of chol and CDC acids combined durin the test meal ranged from 0.41 t 1.48 mM. The ratio typically showed greater concentration of chol acid compared to CDC acid
[Signer *et al.,* 1974]	18 healthy preterm neonates (32-39 weeks)	2 hours after last feed	Duodenal	0.44-23.3 mM Mean value = 6.32 mM	Not stated.

[Challacombe *et al.*,1975]	34 neonates/infants (birth-7 months)	2 hours after last meal	Duodenal	1.65 ±1.1 mM	Glycine/taurine conjugates 0.0 (±0.03) mM Trihydroxy/dihydroxy bile acid 1.8 (±1.3) mM TC acid 0.78 (±0.36) mM TCDC acid 0.68 (±0.40) mM TLC acid 0.32 (±0.17) mM GC acid 0.25 (±0.15) mM GCDC acid 0.55 mM (1 sample)
[Brueon *et al.,* 1978]	20 low birth weight neonates (12-22 days)	3 hours after last meal	Duodenal	3.2-6.9 mM	Glycine conjugates 1.2-4.6 mM Taurine conjugates 0.9-2.3 mM
[Glasoge *et al.,* 1980]	36 neonates (34 ±2.6 weeks) 16 infants/children (25 ±21 months)	Pre-prandial sample	Duodenal	~3-4 mM (neonates) ~5-7 mM (infants/children) Data sets read from graph and exact values not available	GCDC formed 11% of total bil salts in neonates TLC was detected in highe frequency in the infant/childre group compared to neonates
[Jarvenpaa *et al.,* 1983]	66 healthy preterm neonates (33-36 weeks)	1-3 hours after last feed	Duodenal	Median value 3.63 mM in formula fed neonates Median value 7.56 mM in breast fed neonates	High levels of 2-OH cholate bil acids; CDC
[Boehm *et al.,* 1991]	42 low birthweight neonate/infants (15-51 days)	Pre-prandial sample	Duodenal	4.60 ±2.51 mM	No details on composition of bil acids
[Boehm *et al.,* 1997]	41 healthy preterm neonates/infants (8-58 days)	3-4 hours after last meal	Duodenal	27-28 gestational weeks: 4.25 ±2.07 mM 33-34 gestational weeks: 4.47 ±2.10 mM	Secondary bile acids were no detectable Cholic acid; CDC acid; DC acid an LC acid were present
[Van den Abeele *et al.,* 2018]	11 neonates (0-28 days) 3 infants (28 days- 2 years) 30 children (2-12 years) 10 adolescents (12-18 years)	Pre-prandial	Gastric	0.0-5.60 mM 0.0-1.61 mM 0.0-1.11 mM 0.0-6.28 mM	In neonate and infar populations the relative order G > TC > TCDC > GCDC In children and adolescent where the order was GC > GCD > TC > TCDC > GDC > TDC > GUD

7.9 Materials and Methods

7.9.1 Source of intestinal fluid samples

All samples were collected from patients at Birmingham Children's Hospital, a large teaching hospital that is part of Birmingham Women's and Children's Hospital NHS Foundation Trust, UK. Ethical approval was granted by South Birmingham NRES Committee (IRAS 251909). Gastric and intestinal fluid samples were collected from participants during routine clinical endoscopy. Clinical protocols requested that no fluid was ingested in the 90 minutes prior to the endoscopy procedure. Gastric samples were collected from the gastric antrum and intestinal samples from the duodenum. The samples were stored at -80°C prior to characterisation. The participants were stratified by age into the following groups, based on the International Conference on Harmonization (ICH) E11 classifications: < 2 years: new-born/ infant/ toddler (the term infant is used for this group for the remainder of this manuscript), 2-5 years: pre-school age children, 6-11 years: school age children, 12-16 years: adolescents.

7.9.2 Chemicals

Bile salt standards: Cholic acid (CA); Glycocholic acid (GC); glycochenodeoxycholic acid (GCDC); glycodeoxycholic acid (GDC); glycoursodeoxycholic acid (GUDC); taurocholic acid (TC); taurochenodeoxycholic acid (TCDC); taurodeoxycholic acid (TDC); tauroursodeoxycholic acid (TUDC); taurolithocholic acid (TLC); deoxycholic acid (DC), lithocholic acid (LC), and ursodeoxycholic acid (UDC) were purchased from either Sigma Aldrich (Gillingham, UK) or Acros Organics (Fisher Scientific, Loughborough, UK). Internal standards (IS) were specific isotope labelled standards of cholic acid-D4 (D4-CA) and deoxycholic acid-D4 (D4-DC), purchased from Sigma Aldrich. Further details of physchem properties, CAS number, % purity and purchase details of all standards are provided in Table 2 in the supplementary information.

7.9.3 Methodology for characterisation of fluid samples collected

7.9.3.1 pH

A Hanna HI 2210 pH meter was used for all measurements, calibrated on the day of use. A narrow pH electrode (Hanna HI1331B) was used to enable measurement of the small volumes available.

7.9.3.2 Buffer Capacity (mmol/L)

The buffer capacity was measured by titrating each sample with 0.1M NaOH under constant stirring whilst monitoring the pH to measure the volume required for a change in pH of 1 unit. A calibrated Hanna HI 2210 pH meter was used for all measurements. Previous studies measured buffer capacity using both NaOH and HCl, however due to the small sample volumes available, only titration against NaOH was performed for all samples [Litou *et al.,* 2020]. The buffering capacity (β) was calculated using the Equation 7 1:

Equation 7 1

$$\boldsymbol{\beta = \frac{\Delta A}{\Delta pH}}$$

Where ΔA is the amount of acid added and ΔpH is the change in pH induced by the acid added.

7.9.3.3 Osmolality (mOsm/ kg)

Osmolality was measured using a freezing point Osmomat 3000 that was calibrated prior to use. 50 µl of each fluid sample was placed into the appropriate sample vial using a 20-200 µl Thermoscientific pipette and the osmolality value was recorded.

7.9.3.4 Quantification and identification of bile salts

A LC-MS/MS method was used based on published literature [Riethorst *et al*., 2016]. Separation of 14 bile acids was achieved using a dual pump Shimadzu LC-20AB Prominence liquid chromatograph equipped with SIL-20A autosampler, a DGU-20A3 vacuum degasser and an Ascentis Express C_{18} column (15 cm x 4.6 mm I.D., 2.7 µm; Sigma Aldrich). A mobile phase program based upon (A) 1:1 methanol/water and (B) methanol at a flow rate of 150 µL/min was applied for elution of the analytes. Both solvent's pH was adjusted to 9.0 with 0.1% ammonium hydroxide (25%) and 10 mM/L ammonium acetate. The flow was started at 50% B and increased to 100% at 4 minutes, held at 100% B for 5 minutes and reduced to 50% at 12 minutes.

Mass spectrometric analysis was performed using a Sciex API 2000 triple quadrupole mass spectrometer operated in electrospray negative ionization mode. MS/MS detection operated in the multiple reaction monitoring (MRM) mode was used for quantitative determination based on m/z full details are provided in Table 3 of the supplementary material.

Sample preparation: A simple protein precipitation method was followed for extraction of all bile acids from gastric and duodenal fluids. An aliquot of 100-250 µL fluid sample was

precipitated with 440 µL of acetonitrile: methanol (1:2) solvent containing 10 µL internal standard (IS) (1000 ng/mL) and mixed for 15s on a Vortex Mixer (Fisherbrand, UK). This sample mixture was centrifuged at 14,000 rpm at 10°C for 10 min. Initially the samples were diluted 2 times and then 5, 10, 100, and 200 times depending on the concentrations of each bile salt in the fluid sample. From the diluted supernatant 5 µL was injected onto the LC-MS/MS system for analysis.

A simple protein precipitation extraction technique was sufficient to obtain the best recovery for both the bile acid analytes and internal standards. The results of the comparison of neat standards (methanol: water spiked with bile acids) versus surrogate-matrix extracted standards for all the bile acids and the mean recovery was found to between 95-98% at three concentrations (5, 100 & 2000 ng/ml). The recovery for internal standards at 1000 ng/ml was > 98% in all the recovery samples.

The analysis method was shown to be linear from 2-2000 ng/mL and was capable of accurately and precisely determining bile acid concentrations in GI fluid samples according to the FDA requirements for bioanalytical method validation. Total bile acid concentration in each sample was calculated as the sum of the concentrations of the individual bile acids.

7.10 Description of Statistical Methods

Previous work to characterise the gastric and intestinal fluids in adults reported means, medians and range values of pH; buffering capacity; osmolality; viscosity and bile salt concentration [Lindahl *et al.,* 1997]. This work aims to characterise the same parameters for paediatric populations and to explore whether the values obtained are statistically similar to those reported in previous studies in both adult and paediatric populations. The differences in mean values between the sub-sets of paediatric populations as well as existing data from adults were compared using ANOVA analysis (with Tukey's post-hoc) to determine any significant differences. Outliers were identified using SPSS, these are presented in figures but were excluded from further analysis (SPSS uses a step of 1.5×IQR (Interquartile range) to identify outliers).

7.11 Results and Discussion

7.11.1 Patient demographics

A total of 55 children were recruited to the study ranging in age from 11 months to 15 years old. A total of 53 gastric fluid samples were collected with 2 from infants; 10 from pre-school

aage children, 20 from school age children and 21 from adolescents. A total of 40 intestinal fluid samples were collected with 2 from neonates-infants; 7 from pre-school age children; 16 from school age children and 15 from adolescents. Demographic data for all participants is provided in Table 7 4.

Table 7 4 Demographics of the participants included in the fluid characterisation study

Participant identification code	Ethnicity	Age (y)	Height (cm)	Weight (Kg)	Reason for endoscopy	Final diagnosis following endoscopy	Gastric fluid sample	Duodenal fluid sample
UK001	White British	13	158.6	48.5	Bleeding per rectum	Colonic polyp	Yes	
UK002	Any other ethnic group, not specified	10	142	33.2	Abdominal pain	Normal	Yes	Yes
UK003	White-not specified	13	173	56.2	Diarrhoea + Anaemia	Coeliac Disease	Yes	
UK004	Not specified	2	84.1	11.9	Vomiting	Eosinophilic oesophagitis	Yes	
UK005	Asian/Asian British - Indian	11	151	38	Abdominal pain	Normal	Yes	Yes
UK006	White British	8	134.9	44.3	Vomiting	Normal	Yes	Yes
UK007	White British	6	112.8	21	Vomiting	Normal	Yes	Yes
UK008	White-not specified	2	94.2	14.9	Diarrhoea	Normal	Yes	
UK010	White British	12	155.4	45.3	Abdominal pain + constipation	Normal	Yes	Yes
UK011	Black/Black	14	166.6	57.4	Abdominal pain	Normal	Yes	

	British - any other black background							
UK012	White British	15	154	40.7	Abdominal pain	Crohn's Disease	Yes	
UK013	Asian/Asian British - Pakistani	11	147.5	66.6	History of choking episodes	Normal	Yes	Yes
UK014	Mixed White and Black Caribbean	14	169.9	79.9	Abdominal pain	Normal	Yes	Yes
UK015	White British	14	171	62.8	Abdominal pain + Diarrhoea	Normal	Yes	
UK017	White British	14	152.6	52.5	Dyspepsia	Normal	Yes	Yes
UK018	White British	12	169	60.9	Abdominal pain	Normal	Yes	Yes
UK019	Asian/Asian British	15	163.5	37.8	Abdominal pain, diarrhoea + weight loss	Crohn's Disease	Yes	Yes
UK020	Not specified	3	91.8	15.1	Asymptomatic type 1 diabetic patient, screening for Coeliac Disease	Coeliac Disease	Yes	
UK021	Asian/Asian British - Indian	3	94.9	14.3	Hypothyroidism-routine screening for Coeliac Disease	Coeliac Disease	Yes	Yes

UK022	Asian/Asian British - Pakistani	11	139	22.5	Slow weight gain	Coeliac Disease	Yes	Yes
UK023	Not specified	15	177.1	64.2	Abdominal pain	Normal	Yes	Yes
UK024	White British	11	158	38.2	Abdominal pain	Normal	Yes	Yes
UK025	White British	12	162.9	52.2	Abdominal pain + weight loss	Normal	Yes	Yes
UK026	Not specified	13	150.2	42.1	Abdominal pain	Normal	Yes	Yes
UK027	Asian/Asian British - Indian	7	129	22.5	Difficulty in swallowing	Eosinophilic oesophagitis	Yes	
UK028	White British	12	153.7	47.1	Surveillance endoscopy	Eosinophilic oesophagitis	Yes	
UK029	White British	15	179.2	61.8	Abdominal pain	Normal	Yes	Yes
UK030	Asian/Asian British - Pakistani	10	123	21.2	Protein-losing Enteropathy	Normal	Yes	
UK031	White British	9	136.6	25.9	Abdominal pain	Normal	Yes	Yes
UK032	Asian/Asian British - Pakistani	1	77.5	8.5	Part of work up for stem cell transplant	Normal	Yes	Yes
UK033	Asian/Asian British - Indian	15	155.6	39.8	Inflammatory bowel disease surveillance	Normal	Yes	Yes
UK034	White British	15	167.8	47.4	Slow weight gain	Normal	Yes	Yes

UK035	Asian/Asian British - Pakistani	10	130.1	18.6	Abdominal pain, slow weight gain + constipation	Normal	Yes	Yes
UK036	White British	5	117.4	22.6	Suspected Coeliac Disease	Normal	Yes	Yes
UK037	Mixed White and Black Caribbean	6	131.8	35.9	History of diarrhoea	Normal	Yes	Yes
UK038	Asian/Asian British - Indian	8	134	44.1	Surveillance of Reflux Oesophagitis	Reflux Oesophagitis	Yes	Yes
UK039	White British	12	164	57.5	Surveillance of Eosinophilic Oesophagitis	Helicobacter gastritis. Eosinophilic oesophagitis	Yes	Yes
UK040	Mixed other	12	161.9	41.8	Surveillance of Duodenal ulcer	Duodenal ulcer	Yes	Yes
UK041	White British	12	141.3	29.9	Surveillance of Crohn's Disease	Crohn's Disease	Yes	Yes
UK042	White British	3	99.7	14.4	History of blood in vomit	Normal	Yes	Yes
UK043	Asian/Asian British - Indian	11	144.1	31.8	Suspected Inflammatory Bowel Disease	Ulcerative Colitis	Yes	Yes

UK044	White British	8	119.8	21.9	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK045	White-not specified	14	184	57.6	Abdominal pain	Normal	Yes	Yes
UK046	White British	3	97.1	15.4	Complaints of Diarrhoea	Normal	Yes	Yes
UK047	White British	7	112.6	17.6	Surveillance of Crohn's Disease	Crohn's Disease	Yes	Yes
UK048	White British	6	115.2	19.9	History of diarrhoea	Normal	Yes	Yes
UK049	White British	11 months	65	7.4	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK050	White British	10	129.1	25.5	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK051	White British	10	133.6	28.9	Suspected Coeliac Disease	Normal	Yes	Yes
UK052	Asian/Asian British	6	129	24.1	Surveillance of Ulcerative Colitis	Ulcerative Colitis	Yes	Yes
UK053	White-not specified	4	99.7	16.5	Suspected chronic diarrhoea	Constipation	Yes	Yes
UK054	White British	2	88.3	14.1	Bloody diarrhoea, suspected inflammatory bowel disease	Ulcerative Colitis	Yes	Yes
UK055	Asian/Asian British - Indian	4	109.1	18.2	Abdominal pain, suspected coeliac disease	Coeliac Disease	Yes	Yes

UK056	Asian/Asian British - Pakistani	13 months	82	8.6	Not recorded	Not recorded		Yes
UK057	White British	2	83	10.8	Chronic diarrhoea, suspected Coeliac disease	Coeliac Disease	Yes	Yes
UK058	White British	5	110.7	18.4	Chronic diarrhoea	Normal	Yes	Yes
UK059	White British	2	91.8	13.8	Vomiting	Gastro Oesophageal Reflux disease	Yes	Yes
UK060	White British	5	116.9	20.75	Dyspepsia	Functional dyspepsia	Yes	Yes
UK061	Asian/Asian British - Pakistani	3	100.5	14.5	Bloody diarrhoea	Ulcerative Colitis	Yes	
UK062	White British	4	109	19.3	Not recorded	Not recorded	Yes	Yes

Analysis (visual review of data plus statistical analysis where sample sizes permitted this) was undertaken to explore the impact of ethnicity and final diagnosis following endoscopy on all characterisation parameters and no significant correlations were identified. All data was stratified based on age for subsequent data presentation.

7.11.2 pH of gastric and intestinal fluids

The pH values measured are shown stratified by age in Figure 7 1 from 53 participants' gastric fluid and 40 participants' intestinal fluid samples.

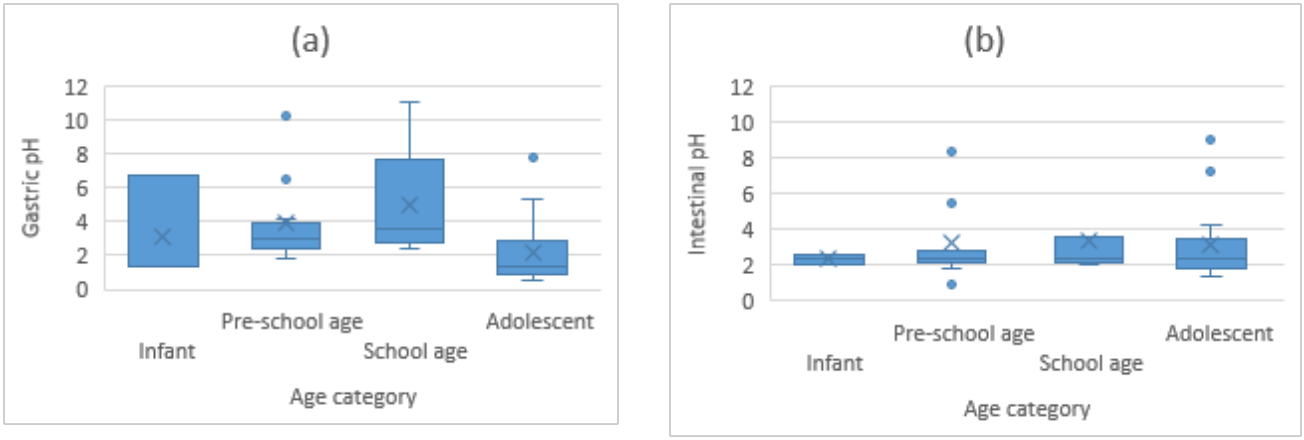


Figure 7 1 Box and whisker plots showing the pH from (a) gastric and (b) intestinal samples stratified by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circle datapoints.

As shown in figure 7 1,there is a lot of variability in the data where the pH values recorded ranged from pH 0.57 to 11.05 (mean: 3.51) in gastric fluids and from 0.89 to 8.97 (mean: 3.15) in intestinal fluids. Statistically significant differences in pH values were only identified for gastric samples between the school age and adolescent aged sub-groups (p=0.013). Previous reports on pH measurements of aspirated fluids from both paediatric and adult populations have also reported large variability [Pedersen *et al*., 2000; Van den Abeele *et al.*, 2018]. The median, mean and standard deviation are shown by age in Table 7 5.

Some of the very high pH values measured may be an artefact of the measuring technique where the pH probe was not measuring a homogenous aqueous liquid and there was some form of physical interference by the other components, for example, solid materials or oils within the aspirated fluid that prevented an accurate measurement. Previous data sets have reported higher than expected pH values within gastric fluids in paediatric populations [Van den Abeele *et al.,* 2018]. However, outliers, which included those with a gastric pH>10, were excluded from subsequent analysis (as described in the methods section).

Table 7 5 Median, mean and standard deviation of the pH of gastric and intestinal samples. Note that outliers were excluded from this analysis.

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	3	13	12	18
	Median	1.36	2.77	3.24	1.31
	Mean	3.14	2.78	4.06	1.67
	Standard deviation	3.09	0.61	2.05	1.15
Intestinal fluid	Number of samples	2	13	6	13
	Median	-	2.36	2.29	2.26
	Mean	2.30	2.23	2.49	2.38
	Standard deviation	-	0.48	0.58	0.81

The gastric pH was higher in this study than previously reported in adults and paediatric studies. The samples in the current study were taken during endoscopy where the children were under a general anaesthetic. Under anaesthetic there may be relaxation of the pyloric sphincter and thus mixing of gastric and intestinal fluids which can affect the pH. Depression of protective reflexes during anaesthesia and loss of consciousness has been reported to predispose patients to, duodenal-gastric reflux, specifically those with abdominal pain [Szarszewski *et al.,* 1999]. This factor this may explain the increased mixing between intestinal and gastric fluids within this patient population. It is worth noting that previous studies characterising paediatric gastric fluids obtained the fluid samples via indwelling nasogastric tubes rather than under anaesthetic [Van den Abeele *et al*., 2018].

The mean values for gastric pH in school age children and adolescents matches previously reported values pH<3 in the literature [Van den Abeele *et al.,* 2018; Fallingborg *et al*., 1990; Schmidt *et al.,* 2015]. Although previous studies [Van den Abeele *et al.,* 2018; Fallingborg *et al*., 1990; Schmidt *et al*., 2015] have reported that children have gastric pH values of less than 3; Van den Abeele *et al* reported that 3/35 gastric samples from children were greater than pH 4 whereas in our study 11/49 were higher than pH 4 thus there was an increased frequency of higher pH values in our study compared to previous data. As stated previously this difference may be linked to the methodology associated with collection of fluids.

Previous literature (shown in Table 7 6) reported mean or median intestinal fluid pH values in children to range from 6-8 which is higher than the values identified in this study. Our values are also lower than reported values from a review of studies conducted in adults where mean values ranged from 5.7-7.5 [Fuchs *et al.,* 2014]. These lower than anticipated values are again

likely to be a result of the mixing of gastric and duodenal fluids due to the sampling technique where the participants were anaesthetised prior to the endoscopy. The samples were frozen prior to measurement of pH as immediate measurement was not possible. Previous studies have reported that pH values can drift over time when samples are exposed to laboratory conditions due to transformation of bicarbonates to carbon dioxide which may also contribute to the higher than expected values for the gastric and lower for the intestinal fluids [Kalatzi *et al*., 2006].

Table 7 6 Reported intestinal pH values from studies conducted in children

Reference	Sample details	Intestinal pH value recorded
[Krafte- Jacobs *et al.,* 1996]	In situ pH measurements from 34 children <4 years old	Mean pH of 6.6 Range 5.6-7.3
[Gharpure *et al.,* 2000]	Intestinal aspirates from 35 children (8 days – 19 years) In a subset of fasted children (n=25)	Mean pH of 6.8 Range 4.5-12.0 Mean pH of 7.0 Range 5.5-7.6
[Westhus *et al.,* 2004]	Intestinal aspirates from 7 children (0-14 years)	Mean pH 7.5 Range 5.9-8.2
[Metheny *et al.,* 1999]	Intestinal aspirates from 2 infants	Mean pH 7.8 Range 7.39-8.20
[Fallingborg *et al*., 1990]	In situ pH measurements from 12 children aged 8-14 years	Mean value of 6.4 (duodenum)

7.11.3 Buffer Capacity

Buffer capacity measurements were obtained from 52 gastric and 38 intestinal fluids and the results are shown in Figure 7 2. The measured buffer capacity (mmol/L/ΔpH) ranged from 0-189 and 5-150 for gastric and intestinal fluids respectively. There was no effect of age upon buffer capacity, although higher gastric buffer capacity values were recorded for older children.

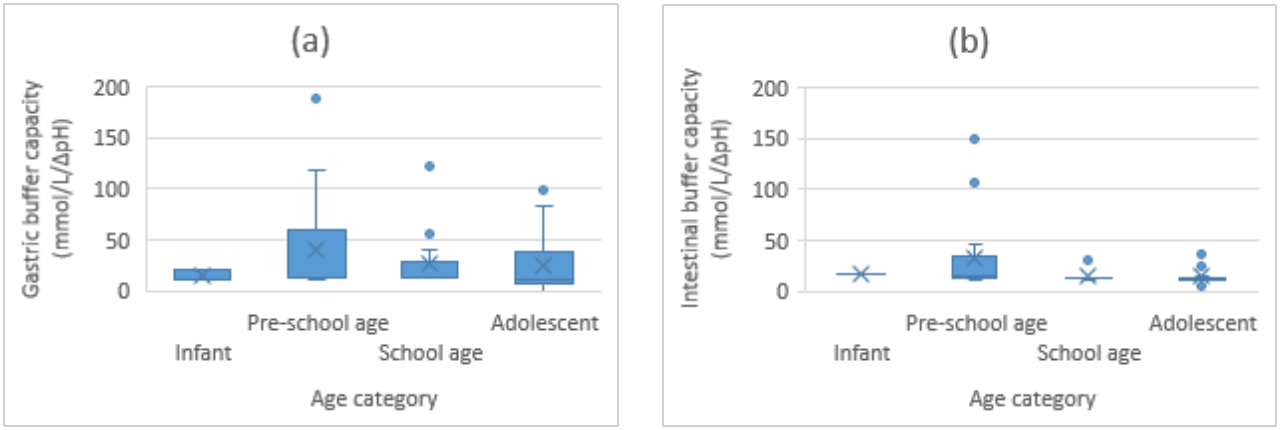


Figure 7 2 Box and whisker plots showing the buffer capacity (mmol/L/ΔpH) for (a) gastric and (b) intestinal samples by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circles.

Following removal of outliers the median, mean and standard deviation were calculated and are presented in Table 7 7. No statistically significant differences were found in buffer capacity based on age sub groups.

Table 7 7 Median, mean and standard deviation of the buffer capacity of gastric and intestinal samples. Note that outliers were excluded from this analysis.

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	3	16	14	15
	Median	10.00	13.00	13.50	10.00
	Mean	13.67	39.94	26.29	11.71
	Standard deviation	6.35	52.61	30.19	11.12
Intestinal fluid	Number of samples	1	14	5	11
	Median	17.00	13.00	12.00	12.00
	Mean	17.00	18.57	12.20	12.09
	Standard deviation	-	10.86	0.45	0.94

The buffer capacity did not change significantly between gastric and intestinal fluids, this could be attributed to the fact that pylorus sphincter is relaxed due to anaesthesia causing the gastric and upper intestinal contents to be mixed.

The buffer capacity of intestinal fluids is of interest as changes in the pH can have dramatic effects on API solubility, thus low buffer capacity can indicate a risk of a change in the pH due

to dissolution of an API or excipients. Previous literature has reported buffer capacity values for fasted adult human intestinal fluids ranging from 2.5-13 mM/L/ΔpH [Fuchs *et al*., 2014; Kalantzi *et al*., 2006]. Higher buffer capacity has been reported in the fed state and also following ingestion of water [Kalantzi *et al*., 2006]. No data has been identified that reports the buffer capacity of paediatric fluids. The existing proposed paediatric simulated intestinal fluids have a buffer capacity of 10 mM/L/ΔpH [Maharaj *et al.,* 2016] which matches the median values from our data as well as the buffer capacity of adult FaSSIF which is 12 mM/L/ΔpH) [Jantratid *et al*., 2008].

7.11.4 Osmolality

Osmolality measurements were obtained from 52 gastric and 40 intestinal fluids and the results are shown in Figure 7 3.

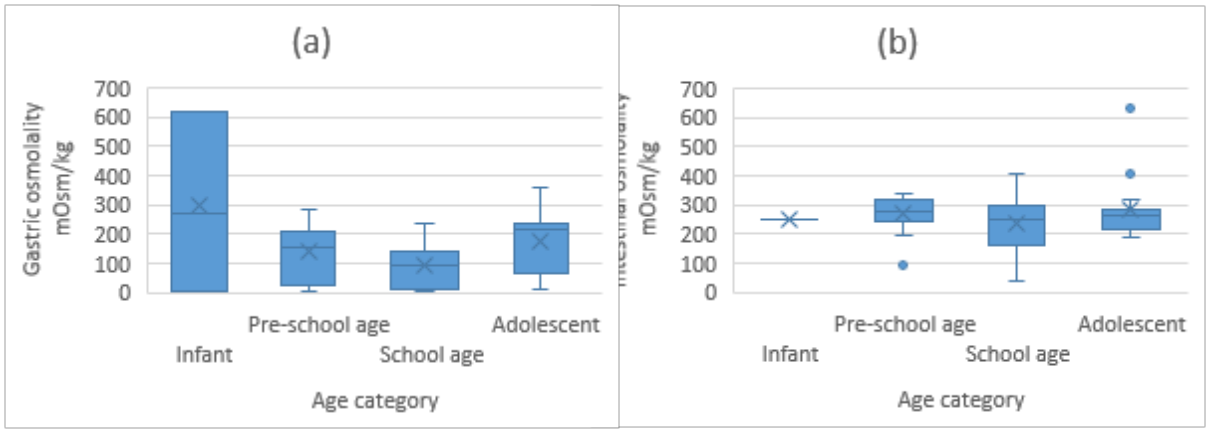


Figure 7 3 Box and whisker plots showing the osmolality for (a) gastric and (b) intestinal samples by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circles

The data generated in this study showed that gastric fluid osmolality values ranged from 1 to 615 mOsm/kg, while intestinal fluid values ranged from 35 to 631 mOsm/kg. Outliers, as identified in Figure 7 3, were excluded prior to statistical analysis and the median, mean and standard deviation for each age group are shown in table 7 8. Statistically significant differences were observed between the gastric osmolality in infants compared to that in school age children, however the variability was large for infants and the sample size small thus caution is required with the interpretation of this data.

Table 7 8 Median, mean and standard deviation of the osmolality of gastric and intestinal samples. Note that outliers were excluded from this analysis.

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	3	16	14	20
	Median	272.00	154.50	91.00	216.00
	Mean	296.33	137.50	91.79	176.75
	Standard deviation	307.22	96.65	71.79	100.87
Intestinal fluid	Number of samples	2	15	5	14
	Median	-	283.00	246.00	260.50
	Mean	248.00	280.47	237.60	248.29
	Standard deviation	-	42.24	51.22	36.58

There is limited data available on osmolality in paediatric populations. Previous reports from aspirate d gastric fluids from children include mean values of 253 mOsm/L from a sample of 40 children under 2 years of age [Wakayama *et al*., 1998] and median values of 274 mOsm/kg for neonates; 188 mOsm/kg for children aged 2-12 years and 219 mOsm/kg for adolescents (12-18 years) [Van den Abeele *et al*., 2018]. Note that some studies reported osmolarity (per litre) whereas others report osmolality (per kg), these units can be interchanged if we assume that the density of the carrier fluid is 1kg/L. Thus, the data obtained in our study shows relatively low values for gastric osmolality in comparison with previous studies. The presence of food components or digested food is likely to have a major effect on the osmolality of gastric fluids and is acknowledged as a potential limitation by other similar studies [Van den Abeele *et al.,* 2018], our samples were from fasted patients which may explain our lower values.

Published data on osmolality of intestinal fluids in children was not identified, however, previous literature has reported osmolality values of 137-299 mOsm/kg in aspirated intestinal fluid from adults [Fuchs and Dressman, 2014]. Thus, the osmolality values measured within this study are within the range previously reported for adults.

The osmolality of the current paediatric fasted state simulated gastric fluid proposed by [Maharaj *et al*., 2016] is 120.7 mOsm/kg and our mean data (stratified by age) ranges from 91-216 mOsm/kg which encompasses this value. However, paediatric fasted state simulated intestinal fluid has an osmolality of 180 mOsm/kg which is somewhat lower than the values measured in our study [Maharaj *et al.,* 2016].

7.11.5 Quantification of bile acids

Bile acid concentrations were determined from 47 gastric fluid samples and 27 intestinal fluid samples; both gastric and intestinal samples were available for 25 individuals. The concentrations of bile acids from gastric and intestinal fluids by age of participant are shown in Figure 7 4 and the summary data shown in Table 7 9

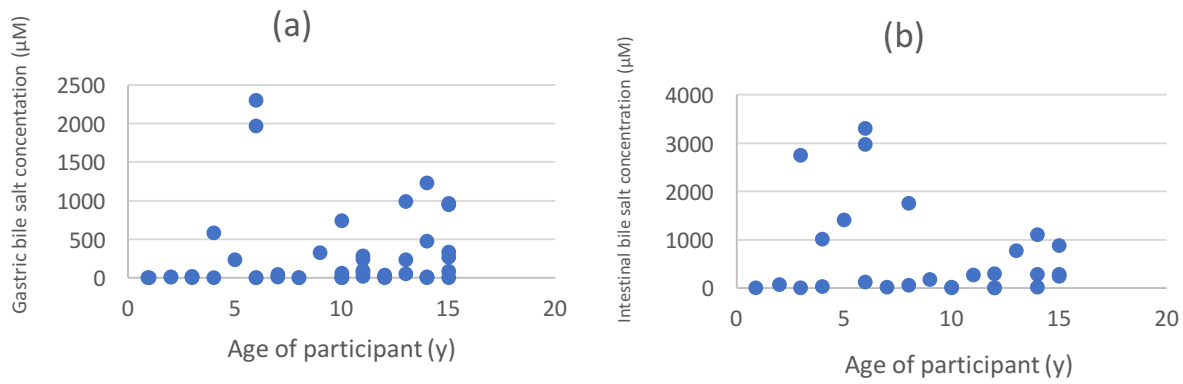


Figure 7 4 Total bile salt concentration (µM) plotted versus the age of the participant in the (a) gastric and (b) intestinal fluids.

Table 7 9 Median, mean and standard deviation of the bile salt concentration (μM) of gastric and intestinal samples.

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	2	8	19	18
	Median	-	9.59	40.23	65.69
	Mean	4.65	108.25	325.24	314.11
	Standard deviation	2.07	207.66	663.87	420.76
Intestinal fluid	Number of samples	1	6	10	10
	Median	0.81	537.37	149.80	277.34
	Mean	0.81	876.81	866.18	386.26
	Standard deviation	-	1088.88	1308.14	393.84

The quantitative analysis of bile salts in both gastric and duodenal fluids (Figure 7 4) showed large variability among subjects and the summary of median, mean and standard deviation are presented in Table 7 9. The outliers were not excluded in this analysis as the variability as well as mean data is of interest for this population. These variabilities could be due to the presence of solid remnants in aspirated fluids. Some of the gastric samples collected were found to be transparent fluid and some contained suspended greenish floccules. It was observed that typically transparent fluids showed lower bile acid concentrations although this was not statistically significant. The total concentration of bile acids in gastric fluid ranged from 0.002-2.3 mM and in intestinal fluid from 0.0008-3.3 mM. These ranges are similar for those previously reported where large variability was also reported. However, the mean values are much lower than values in previous studies where mean values were typically greater than 2 mM. This lower mean value may have implications for the solubilising capacity in paediatric populations as the critical micelle concentration for bile is often reported to be greater than 2mM [Subuddhi and Mishra, 2007].

There was no statistically significant difference in the bile acid concentrations with the age of the participants although there was a trend towards an increased mean concentration in the gastric fluid with increasing age. The large variability and small age-stratified sample sizes from intestinal fluids limit the interpretation of the data. Higher mean and median bile acid concentrations were found in the intestinal samples compared to the gastric samples.

However, as stated previously there may be some mixing of these fluids during the endoscopy thus it was not appropriate to pool samples from the same individual.

The concentration of bile is typically lower in the stomach compared to the small intestine, as reflected in the concentration of bile salts in FaSSGF at 0.08mM whilst the concentration in FaSSIF is 3mM [Jantratid *et al*., 2008]. Proposed paediatric fasted state simulated gastric fluids reported a bile salt concentration of 0.02mM for neonates and 0.06mM for infants which is higher than that reported in our work yet still lower than that in adults which is supported by our data [Maharaj *et al.,* 2016]. Due to the large variability in reported data two bile salt concentrations were proposed for paediatric FaSSIF to account for a minimum and maximum. These were 1.5mM and 4.5mM [Maharaj *et al*., 2016]. However, the bile acid concentrations measured in intestinal fluid in this study are lower and <1 mM in the majority of samples.

The relative contribution of individual bile acids to the total bile concentration was determined and the data are shown by individual sample in ascending order of age in

Figure 7 5. No trends were identified that linked bile acid concentration to age, thus the data were pooled for gastric and intestinal samples to determine the median, mean and standard deviation of each component identified, the data are presented in Table 7 10.

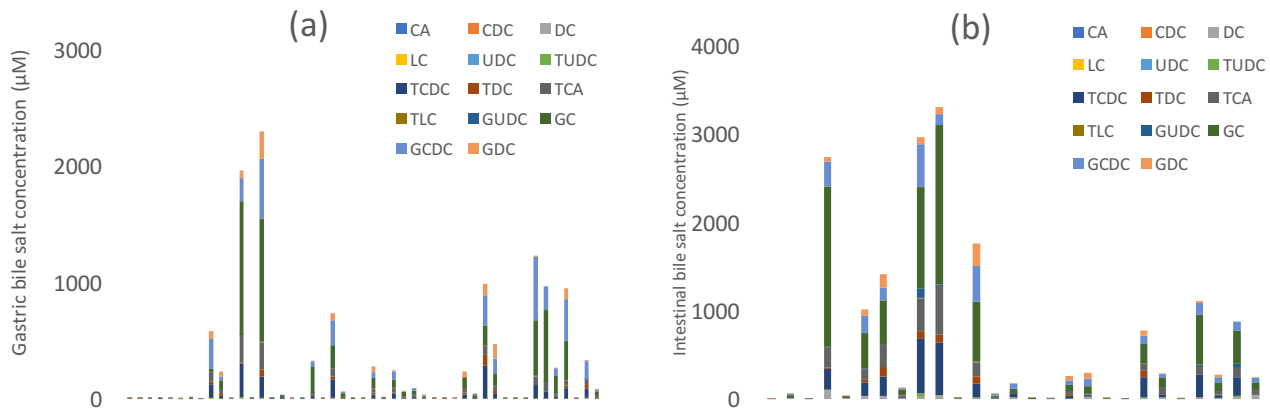


Figure 7 5 Relative contribution of bile salts to the total bile salt concentration in (a) gastric and (b) intestinal individual samples ordered from youngest to oldest within each population.

Table 7 10 . Median, mean and standard deviation of the concentrations (μM) of bile acids present in the gastric or intestinal fluid samples from children.

		Concentration (μM)													
		Primary bile acids		Secondary bile acids			Taurine Conjugated bile acids					Glycine Conjugated bile acids			
		CA	CDC	DC	LC	UDC	TUDC	TCDC	TDC	TC	TLC	GUDC	GC	GCDC	GDC
Gastric fluid (n=47 samples)	Median	0.125	1.549	1.783	0.510	0.000	0.000	1.041	0.146	1.671	0.032	0.075	5.660	3.449	0.582
	Mean	0.148	1.729	1.918	0.576	0.004	0.937	34.547	7.623	26.283	0.417	0.945	107.006	68.056	20.402
	Standard deviation	0.166	0.745	0.767	0.276	0.029	1.918	67.976	17.643	50.879	1.053	2.317	246.538	130.247	44.797
	Number where BS was present	45	47	47	47	2	23	46	37	47	26	43	46	47	39
Intestinal fluid (n = 27 samples)	Median	0.138	0.167	13.434	0.025	0.844	0.334	19.248	1.473	23.085	0.135	0.907	66.860	48.150	3.194
	Mean	0.307	0.291	15.947	0.033	0.929	2.749	105.934	19.644	81.860	1.380	7.814	299.974	87.519	34.253
	Standard deviation	0.518	0.266	19.994	0.028	0.993	6.838	170.549	34.789	131.602	2.291	20.865	514.438	124.453	57.432
	Number where BS was present	21	27	27	27	22	20	27	24	27	25	25	27	27	24

In both gastric and intestinal fluids, the most abundant bile acids were glycine conjugated with glycocholate being the most abundant. Primary bile acids were detected in almost all gastric samples as well as glycine conjugated bile acids. UDC was only present in 2/47 gastric fluid samples and TUDC and TLC were present in approximately half of the gastric samples. The order of magnitude (based on mean values) for the presence of bile acids in gastric fluid was GC> GCDC> TCDC> TC> GDC> TDC> DC> CDC> GUDC> TUDC> LC> TLC> CA> UDC. This relative order matches well to the order previously reported by Riethorst *et al* (2016) based on adult gastric fluids [Riethorst *et al.,* 2016]. There are some differences with the relative order reported by Van den Abeele *et al* (2018) yet this may be due to the difference in the ages within the paediatric populations or due to some contamination of gastric samples with intestinal fluids due to the method of sample collection [Van den Abeele *et al.,* 2018].

Most bile acids were detected in the 27 intestinal fluid samples analysed. The taurine conjugated bile acids were present in higher levels compared to within the gastric samples. The order of magnitude (based on mean values) for the presence of bile acids in intestinal fluid was GC> TCDC> GCDC> TC> GDC> TDC> DC> GUDC> TUDC> UDC> CA> CDC> LC. Previous literature (highlighted in Table 7 11) showed similarities to the relative concentrations found in these samples where GC; TC; GCDC and TCDC are the most commonly identified bile acids in paediatric intestinal fluids.

Further work is required to generate understanding on the implications of the range of bile salts present and their relative concentrations on the solubilisation of APIs. Specific work will investigate the impact of bile salt concentration and composition identified within this study on the solubility of a series of drugs previously investigated in adult human intestinal fluids [Heikkila *et al.,* 2011; de la Cruz Moreno *et al.,* 2017] to better understand how solubility may differ in a paediatric population compared to an adult population. Furthermore, the data will be used to drive the development of paediatric relevant fasted state simulated intestinal fluid to integrate into paediatric biopharmaceutics toolkits.

7.12 General Discussion

The data generated from the paediatric gastro-intestinal samples showed large inter-individual variability in all parameters characterised. There were no trends identified when the data was interrogated based on the age, ethnicity or disease-state of the participant. The lack of trends identified may have been masked by the variability observed. The similarities

in the properties of the gastric and intestinal fluids suggests mixing of these fluids during the endoscopy procedure as this was conducted under anaesthetic. Characterisation from indwelling naso-gastric or naso-duodenal tubes would provide a cleaner data set and will be the target for future research.

However, the variability associated with gastro-intestinal fluids is an important finding as this can affect the solubility of drugs within a population. For example, recent work has highlighted that variability in bile acid metabolism as a result of gut microbiota can affect the solubility of a series of drugs [Enright *et al*., 2017]. Thus, it is prudent to develop a suite of bio-relevant media for a paediatric population to reflect this diversity and better understand the potential variability associated with solubility *in vivo* based on differences in the gastro-intestinal environment. The use of the median data to develop a mid-point fluid will provide a single point estimate for solubility whereas this in conjunction with extreme variants will provide understanding on the potential sensitivity to solubility within a highly variable paediatric population.

7.13 Conclusions

This work provides a comprehensive characterisation of gastric and intestinal fluids from a paediatric population. This provides a useful data set to generate simulated media to represent paediatric populations and to compare to existing simulated fluids based on adult data. The differences noted between paediatric and adult fluids justifies the need for additional experimental research to better understand the implications of these differences on drug solubility. It should be noted that there was large variability within the samples and that there was likely to be mixing of gastric and intestinal fluids. Caution is required for interpretation of the data as the mean values do not represent any single individual, therefore media that represent the extreme individual samples as well as the mean are likely to provide greater insights into the impact of fluid attributes on API solubility and dissolution in paediatric populations.

7.14 Acknowledgements

The authors would like to acknowledge all clinical staff involved in the study for their efforts in enrolling patients and collecting samples leading to the generation of the dataset.

Funding: This work was supported by Janssen Research and Development, Belgium.

7.15 Supplementary information for the published paper

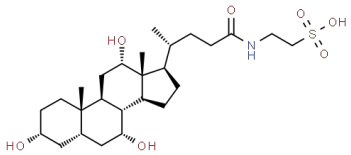
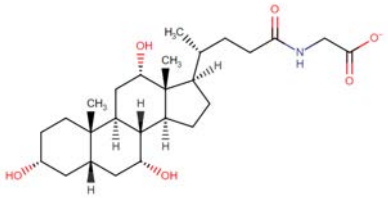
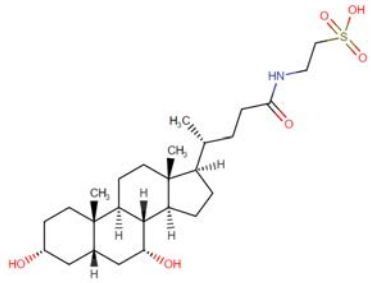
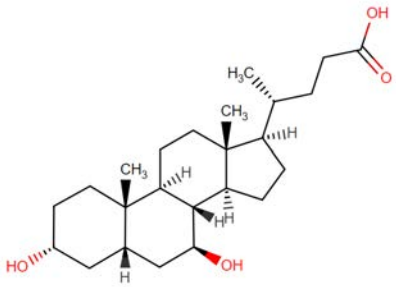
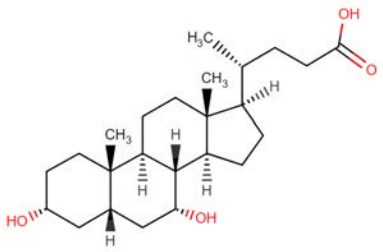
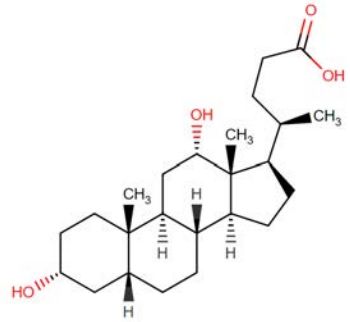
Table 7 11 below presents all studies that characterised either gastric and/ or intestinal fluid and the relevant parameters that were measured.

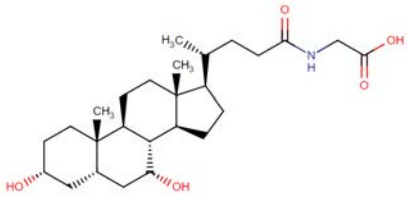
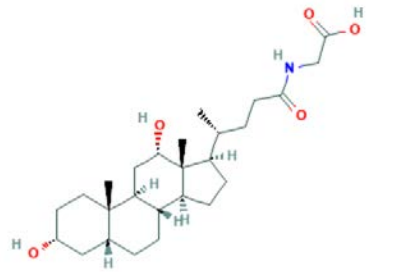
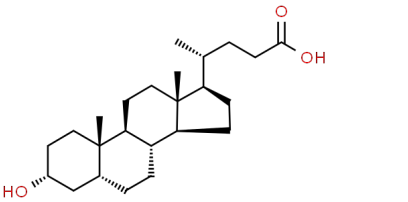
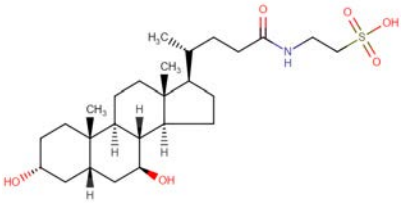
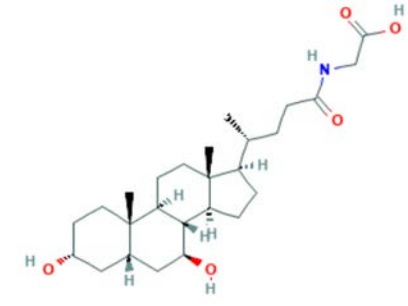
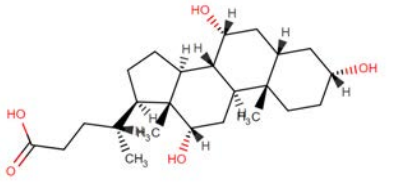
Table 7 11 Summary of cohort details from studies reported where fasted gastrointestinal fluid was collected for characterisation

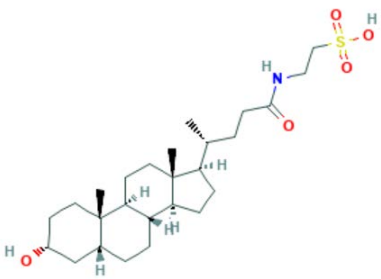
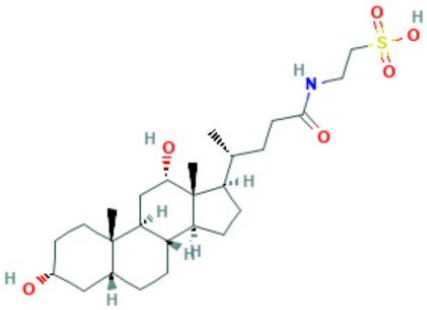
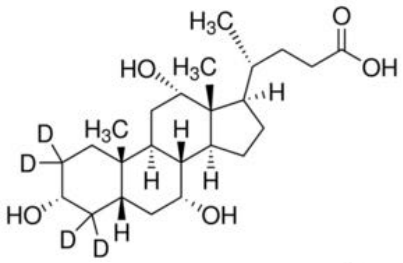
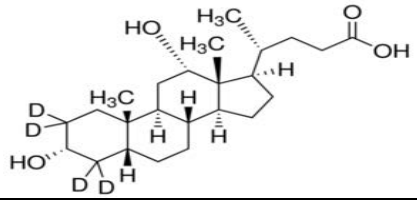
Reference	Population	Fluids characterised	Relevant parameters measured	Co-administration of water
[Lindahl *et al.*, 1997]	24 adult healthy volunteers (19-37 years)	Fasted gastric and jejunal fluid	Bile salt content, pH, osmolality	None
[Pedersen *et al.*, 2000]	9 healthy volunteers (age not reported)	Fasted jejunal fluid	Bile salt content, pH, osmolality	None
[Persson *et al.*, 2005]	12 healthy volunteers aged 24-40	Fasted jejunum	Bile salt content, pH, buffer capacity	None
[Perez de la Cruz Moreno *et al.*, 2006]	6 adult volunteers (22-35 years)	Fasted duodenal and jejunal fluid	Bile salt content, pH, osmolality, buffer capacity	None
[Clarysse *et al.*, 2009]	5 adult healthy volunteers	Fasted duodenal fluid	Bile salt content, pH, osmolality	None
[Heikkila *et al.*, 2011]	5 healthy volunteers (24-39 years)	Fasted duodenal fluid	Bile salt content, pH, osmolality	Sampling followed 15 minutes post ingestion of 250mL water
[Holmstock *et al.*, 2013]	4 healthy volunteers (19-35 years)	Fasted duodenal fluid	Bile salt content, pH, osmolality, viscosity	Sampling followed ingestion of 200mL water
[Foltz *et al.*, 2015]		Fasted gastric fluid	Bile salt content	None
[Riethorst *et al.*, 2016]	20 adult healthy volunteers (18-31 years)	Fasted duodenal fluid	Bile salt content, pH	250mL water administered prior to sampling
[Van den Abeel *et al.*, 2018]	Paediatric (0-18 years)	Fasted gastric fluid	Bile salt content, pH, osmolality	None

Table 7 12 provides information about the bile salts that were used in the analysis, including their chemical structure, molecular weight, logP and water solubility. It is presented as supplementary information to the paper, as there has been work on bile salts' identification and quantification by researcher Gopal Pawar.

Table 7 12 Structure and properties of the bile acid standards used in the analysis.

		weight (g/mol)		solubility		Mol Wt with salt form
Taurocholic acid (TCA)	 $C_{26}H_{45}NO_7S$	515.703	0.79 (ALOGPS)	0.0771 mg/ml (ALOGPS)	Taurocholic acid, sodium salt hydrate, 98%, ACROS Organics (CAS No.-345909-26-4)	98% 555.703
Glycocholic acid (GC)	 $C_{26}H_{42}NO_6$	464.624	1.65	3.3 mg/L (at 20 °C)	Sodium glycocholate Hydrate Sigma-Aldrich (CAS No.-338950-81-5)	≥95% (TLC) 487.60 (anhydrous basis)
Taurochenodeoxycholic acid (TCDC)	 $C_{26}H_{45}NO_6S$	499.704	1.38 (ALOGPS)	0.00748 mg/ml (ALOGPS)	Sodium taurochenodeoxycholate Sigma-Aldrich (CAS No.-6009-98-9)	≥97.0% (TLC) 521.69
Ursodeoxycholic acid (UDC)	 $C_{24}H_{40}O_4$	392.572	3.00	20 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-128-13-2)	≥99%
Chenodeoxycholic acid (CDC)	 $C_{24}H_{40}O_4$	392.572	4.15	89.9 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-474-25-9)	≥96%
Deoxycholic acid (DC)	 $C_{24}H_{40}O_4$	392.572	3.50	43.6 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-474-25-9)	≥98%

Glycochenodeoxycholic acid (GCDC)	 $C_{26}H_{43}NO_5$	449.6233	2.12	3.15 mg/L (at 20 °C)	Sodium glycochenodeoxycholate Sigma-Aldrich (CAS No.-16564-43-5)	≥97% (HPLC) 471.61
Glycodeoxycholic acid (GDC)	 $C_{26}H_{43}NO_5$	449.6	4.3	Water Solubility at 25 deg C (mg/L): 17.95	Sodium glycocholate hydrate Sigma-Aldrich (CAS No.-338950-81-5)	≥95% (TLC) 487.60 (anhydrous basis)
Lithocholic acid (LC)	 $C_{24}H_{40}O_3$	376.573	8.263	Water <1 mg/ml	Sigma-Aldrich (CAS No.-434-13-9)	≥95%
Tauroursodeoxycholic acid (TUDC)	 $C_{26}H_{45}NO_6S$	499.7	1.38	0.00748 mg/ml	Tauroursodeoxycholic Acid, Sodium Salt Sigma-Aldrich (CAS No- 1180-95-6)	≥95% 521.69
Glycoursodeoxy cholic acid (GUDC)	 $C_{26}H_{43}NO_5$	449.6	4.3 (XLogP3)	0.00135 mg/ml	Sigma-Aldrich (CAS No.-64480-66-6)	≥96.0% (TLC)
Cholic acid (CA)		408.5714	-3.37	175 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-81-25-4)	≥98%

	$C_{24}H_{40}O_5$					
Taurolithocholic acid (TLC)		483.7	4.9 (XLogP3)	25 mg/ml	Sodium taurolithocholate (CAS No.-6042-32-6)	≥97.0% (TLC)
Taurodeoxycholic acid (TDC)		499.7	3.6 (XLogP3-AA)	41 mg/ml	Sodium taurodeoxycholate hydrate (CAS No- 207737-97-1)	≥95% (HPLC) 521.69 (anhydrous basis)
Cholic acid-D4 (Internal standard)		412.60			Sigma Aldrich (CAS No.-116380-66-6) 100 µg/mL in methanol	98 atom % D, 98% (CP)
Deoxycholic acid-D4 (Internal standard)		396.60			Sigma Aldrich (CAS No.-112076-61-6) 100 µg/mL in methanol	≥98 atom % D, ≥98% (CP)

7.15 Supporting Information for the thesis

The following section outlines the main methodology presented in the published article and provides additional detail and justification of methods where required

7.15.1 pH measurement protocol

Instrument: Hanna HI 2210 PH METER.

The instrument was calibrated each day in the morning. Calibration took place with two buffer solutions of pH 4 and 10 respectively. The electrode was immersed in buffers and the research was waiting to see the corresponding value on the pH meter's screen. The probe was cleaned with destilled water between buffers and with Chemgene HLD4L (diluted 1:20 because of biological origin of samples). The probe was also cleaned with Chemgene HLD4L 1:20 at the start of the samples' analysis and at the end of the process.

7.15.2 Buffer capacity measurement protocol

Instrument: Hanna HI 2210 PH METER.

Buffer capacity was measured with pH instrument as described at section The following section outlines the main methodology presented in the published article and provides additional detail and justification of methods where required
7.15.1 pH measurement protocol. Other studies have used both 0.1 M NaOH and O.1 M HCL. However, due to the limited available volumes of the paediatric samples (especially when they origin from the younger age groups as neonates, infants, toddlers etc.), buffer capacity was only be measured by adding 0.1 M NaOH to the samples [Litou *et al.,* 2020]. NaOH was added drop by drop and the change in pH recorded. Buffer capacity was then calculated using the formula below:

Equation 7 2 $\boldsymbol{\beta = \Delta A / p}$H
where ΔA= amount added in the solution that caused the change in the pH and ΔpH= final pH value-initial pH.

7.15.3 Osmolality measurement protocol

Instrument: freezing point Osmomat 3000

50 ul of each sample was inserted in eppendorf tubes using a 20-200 ul thermoscientific pipette. The instrument probe was cleaned with Chemgene HLD4L 1:20 before and after use, and distilled water between of each sample.
When collecting the HIF samples for the scope of the thesis, it has been observed that different samples' appearances were linked with different pathologies. Samples had various appearances, e.g: non-transparent, transparent, with mucous content or without, with blood or without.

7.15.4 Paediatric gastric fluid Samples Images

Figure 7 6 and Figure 7 7 demonstrate the appearance of analysed paediatric gastric samples.

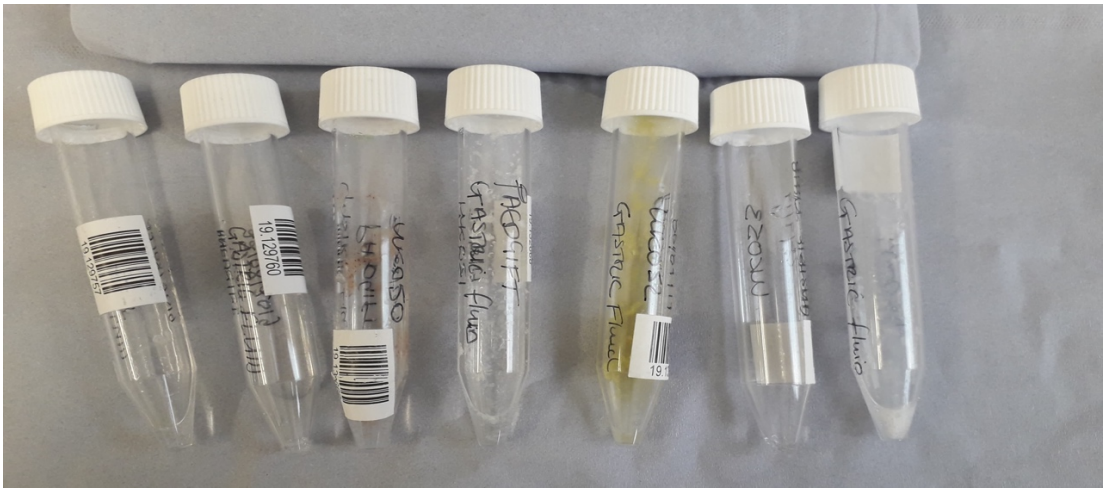


Figure 7 6. Gastric Paediatric Samples prior to the initiation of the analysis

Figure 7 7 Sample to the left: gastric origin, clear, transparent, non-pathological. Sample in the middle: gastric origin, semi-transparent, blood mucosa, coeliac disease. Sample to the right: gastric origin, non-transparent, mucous content, ulcerative colitis

7.15.5 Paediatric intestinal fluid samples images

Figure 7 8 and Figure 7 9 demonstrate the appearance of analysed paediatric intestinal samples

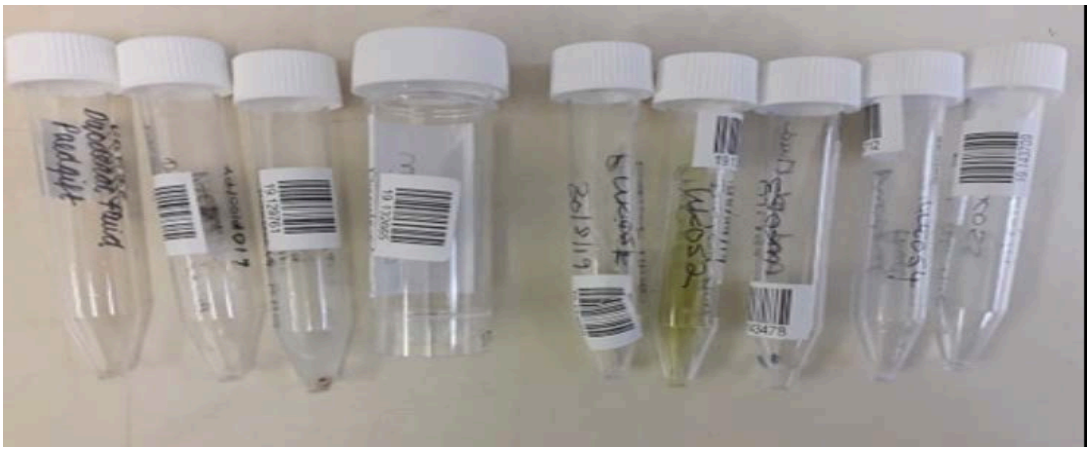


Figure 7 8 Intestinal paediatric samples prior to the initiation of the analysis

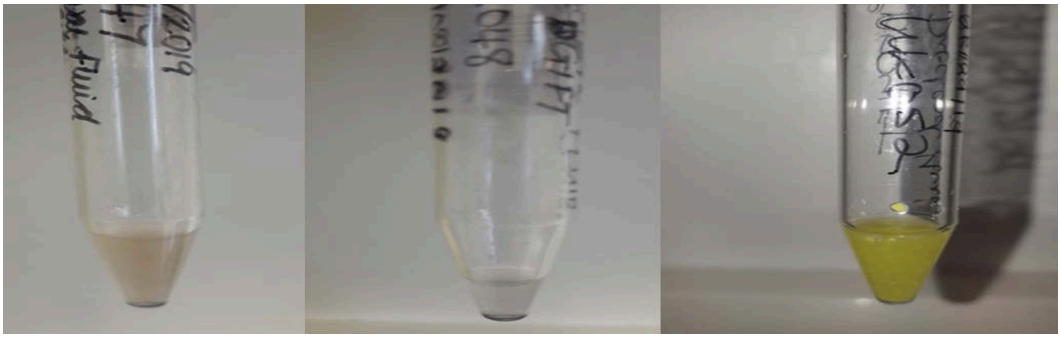


Figure 7 9 Sample to the left: Intestinal origin, clear, non- transparent, Crohn's disease, Sample in the middle: Intestinal origin, transparent, non-pathological, Sample to the right: Gastric origin, non-transparent, mucous content, ulcerative colitis

In both gastric and intestinal samples, appearance had a similar pattern: non-pathological samples were clear and transparent, coeliac disease samples had traces of blood and mucosa, while samples of patients with Ulcerative colitis were non-transparent, yellowish, with mucous content, sample of Crohn's disease: thick, non- transparent.

It is worth noting that the appearance of the samples has not been associated with any of the characteristics measured.

Table 7 13 is an example presenting a link between the pathologies and a dataset of the GI samples.

Table 7 13 Appearance of a dataset of HIF samples.

Patients	Origin	Appearance	Content	Diagnosis
UK025	Gastric	Non-transparent	Mucous content	Ulcerative colitis
UK047	Intestinal	Non-transparent	clear	Crohn's disease
UK048	Intestinal	Transparent	Clear	Normal
UK050	Gastric	Semi-transparent	Blood mucosa	Coeliac disease
UK052	gastric	Non-transparent	Mucous content	Ulcerative Colitis

This thesis aimed to generate new knowledge on paediatric gastrointestinal volumes and fluid composition by exploring the complex paediatric GI tract. The overall aim was to create new paediatric data to update the current *in vitro* and *in silico* tools, and subsequently, predict paediatric oral drug absorption with the maximum accuracy. This would lead to achieving the desired therapeutic effect in the paediatric population.

Chapter 3: Malnutrition chapter has reviewed the impact of malnutrition on the paediatric gastrointestinal tract, and subsequently, on paediatric oral drug absorption. Malnutrition has been vastly neglected as a global health issue and the medicines' efficacy to treat malnourished children has not be given the appropriate attention. It is recognised that malnutrition can alter the GI tract and thus, modify drug absorption. There is evidence that oral cavity, stomach and small intestine is affected by malnutrition in children. The consequences for oral drug absorption have been identified and listed. No information was found with regard to the impact of malnutrition on paediatric colon. To create a complete profile and understanding of malnutrition and its consequences on the paediatric GI anatomy, the colon of malnourished children should be studied and data should become available. The data that were identified within this review, were in general quite old. Thus, there is a need for updating the existing data with new ones.

At the moment, bio relevant *in vitro* tools, that represent the conditions of malnourished children and the different types of malnutrition, are not available. Creating such *in vitro* tools will ensure that the paediatric drug dosages for malnourished children are not only safe, but effective as well.

The experimental work in Chapter 4: Magnetic Resonance Images (MRI) analysis chapter, included collection and analysis of MRI images by a novel MRI method, which is a combination of HOROS and ImageJ softwares. The study included population aged 0-16 years old in fasted and fluid fed state. It was demonstrated that as it was firstly shown for adults [Schiller *et al.,* 2005], water has a discontinuous pattern in the paediatric GI tract as it exists in the form of fluid pockets. The volume and the location of fluid, which freely exists in the paediatric stomach and small intestine, was recorded. Interestingly, the fasted gastric volume in children is considerably lower than that reported in adults in similar studies. This could lead to implications on the disintegration and dissolution of medicines administered to children under fasted conditions. The fasted paediatric gastric volumes in our study are much lower than the fasted paediatric gastric volumes reported

before [Meakin et *al*., 1987; Crawford *et al*., 1990]. This is of interest as it could have an impact on the onset of drug action and limit the therapeutic action of the drug. Additionally, the total number of fluid pockets that was reported with the novel MRI methodology (8 ±4 and 22 ±4 pockets in the fasted and fluid-fed state respectively) was in accordance with the findings in Mudie's *et al.* [2014] study (8 pockets in the adult fasted state and 16 in the adult fed state). Regarding intestinal fluid volumes, our findings showed a fasted mean paediatric small intestinal volume of 7.4 ml, which is considerably lower than values reported previously in adult studies [Schiller *et al*., 2005; Mudie *et al.,* 2014]. The findings regarding the intestinal volumes suggest that the volumes used to represent the intestinal media have to be updated; previously, 50mL for neonates; 100mL for infants and 200mL for pre-school children have been suggested to use [Wollner *et al*., 2018]. These volumes need to be re-considered, as this study suggests that volumes of 7-40mL reflect more accurately the paediatric small intestinal volumes for pre-school and school aged children.

Future work should focus on extending the number of the datasets participating in this study and aim to overcome the limitations of this work; As the datasets derived from children scanned for clinical reasons, the fed state protocol could not be adapted. However, it would be of interest to see the paediatric gastro-intestinal volumes for a fed with solid meal paediatric population and/ or fluid- fed paediatric population with water instead of Oral Klean Prep. Also, it is recognised that children of younger age are unable/ unwilling to consume the exact volume of 500 ml Oral Klean Prep. Therefore, future research should take into consideration that the exact ingested volumes must be recorded, which in this study was not possible due to the retrospective nature of the project.

Chapter 5: Physiological Based Pharmacokinetic Model (PBPK) Model demonstrated that by updating the PBPK model SimCyp paediatric v.18 for ritonavir, with the intestinal volume data of chapter 4, the dosage fraction that is absorbed by the jejunum is increased. This could have an important impact on oral drug absorption. Therefore, it is advised that PBPK models should be updated with the new intestinal volumes to be able to predict with the maximum accuracy the pharmacokinetics of drugs in paediatric population.

Characterisation of paediatric human intestinal and gastric samples took place in

7. Chapter 7: Gastro-intestinal human samples analysis chapter. The samples derived from children that underwent endoscopy in their clinical routine. Characterisation of the fluids included pH, buffer capacity and osmolality measurements. Bile salts have been identified and quantified as well. Many similarities were observed

between the properties of the gastric and intestinal fluids. This was probably attributed to the fact that the endoscopy procedure was conducted under anaesthetic and the pyloric sphincter has relaxed, leading to a partial mixing

of the gastrointestinal contents. Therefore, it is suggested that future research should prefer collecting samples from indwelling naso-gastric or naso-duodenal tubes instead, to avoid the mixing of the contents. Additionally, samples in this study were frozen in the hospital where they have been collected (BCH). However, previous studies have reported that exposing the human gastrointestinal samples to laboratory conditions may cause drifting of pH values over time [Kalatzi *et al.,* 2006]. Future work should focus on conducting immediate measurements where possible, to minimize the effect of bicarbonate transforming to carbon dioxide, which is very likely to have significantly increased the pH of the gastrointestinal samples. Interestingly, our findings have been characterized by large variability. This is of great importance as it can have an effect on the solubility of drugs within a specific population. It is thus of high priority to develop paediatric bio relevant media that will be able to reflect this diversity. Median values do not represent any single individual; on the other hand, extreme values and the mean are able to provide a better understanding of the impact of paediatric gastric and intestinal human samples properties on API solubility and dissolution.

Future research should focus on characterising more paediatric gastro-intestinal samples from neonates, infants, toddlers, in order to have a complete profile of the physicochemical properties of the younger age groups, that have been particularly difficult to recruit. Finally, it is advised that future studies should aim to characterize samples from the healthy paediatric GI tract. This was not possible in this study, as the participants underwent endoscopy for clinical reasons and a great number of them was diagnosed with diseases as coeliac disease, crohn's disease, ulcerative colitis etc. These pathological conditions could have an impact on the physicochemical properties of the samples.

9. References

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