CITRULLINE AND THE INTESTINE

THESIS SUBMITTED BY

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Declaration

I, Konstantinos Fragkos, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Konstantinos Fragkos

April 2018
This thesis is dedicated to the development of greater understanding between health and life sciences and to the opportunity to investigate the complexities of intestinal physiology.
Abstract

Citrulline, a non-protein amino acid, has been playing an important role in scientific research over the last few years. This thesis explores various aspects of citrulline with respect to intestinal disease, short bowel syndrome and intestinal failure. The first important finding was that citrulline as a term has been used at the end of the 19th century-beginning of the 20th century to describe an extract of the *C. colocynthis*, used as a subcutaneous laxative. Also, old sources have revealed that citrulline was first described as an amino acid by Koga and Ohtake (1914) and not by Wada (1930a). From the systematic review and meta-analysis, citrulline levels are strongly positively correlated with small bowel length in short bowel syndrome patients and strongly negatively correlated with intestinal disease severity with regards to enteropathies (coeliac disease, tropical enteropathy, mucositis, acute rejection in intestinal transplantation, but not Crohn’s disease). Citrulline cut-off levels have an overall sensitivity and specificity of 80% and citrulline levels compared to controls were reduced by 10 μmol/L. These findings suggest that citrulline is a marker of possible acute intestinal injury or intestinal insufficiency.

Next, an original five-by-five cross-over study was designed (Williams design) comparing post-absorptive amino acid concentrations after challenges with citrulline, arginine, glutamine, 3-methyl-hisitidine and placebo. Citrulline was the most potent stimulator for all other amino acids, contrary to beliefs of glutamine challenges. Citrulline challenges could be useful in intestinal failure but also in liver failure where urea cycle pathways including glutamine, arginine and ornithine are implicated. The final study was an investigation of quality of life in short bowel syndrome patients. The quality of life scale is highly reliable in short bowel syndrome patients (Cronbach’s alpha > 0.700) and the main causes of low quality of life are fatigue, diarrhoea/increased stomal output, lack of sleep, gastrointestinal symptoms, and muscle pains.
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Publications and Presentations Arising from this Thesis

Publications


Presentations


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**Abbreviations**

3MH/3-MH: 3-Methylhistidine
ADC: Arginine decarboxylase
AMP: Adenosine monophosphate
ANOVA: Analysis of variance
AP-ESI: Atmospheric pressure electrospray ionization
APCI: Atmospheric pressure chemical ionization
ARG/arg: Arginine
ASL: Argininosuccinate lyase
Asp: Aspartate
ASS: Argininosuccinate synthase
ATP: Adenosine triphosphate
AUC: Area under the curve
BMI: Body mass index
C: Celsius
CC: Correlation coefficient
CD: Crohn's disease
CeD: Coeliac disease
CI: Confidence interval
CIT/cit: Citrulline
Cl: Clearance
C\textsubscript{max}: Maximum concentration
CO\textsubscript{2}: Carbon dioxide
CRP: C-Reactive protein
C\textsubscript{t}: Last measured concentration
df: Degrees of freedom
DFBETA: difference in regression coefficients when observation deleted
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
EFA: Exploratory factor analysis
ELISA: Enzyme-linked immunosorbent assay
EMA/EMEA: European Medicines Agency
ESI: Electrospray ionization
F: F-test statistics
FBS: Foetal bovine serum
FDA: Food and Drug Administration
FN: False negative
FP: False positive
GFD: Gluten-free diet
GLN/Gln: Glutamine
GLP-2: Glucagon-like peptide-2
GLU/Glu: Glutamate
HILIC: Hydrophilic interaction liquid chromatography
HIV: Human immunodeficiency virus
HPLC: High performance liquid chromatography
HPN: Home parenteral nutrition
HSROC: Hierarchical summary receiver operating curve
IBD: Inflammatory bowel disease
ICU: Intensive Care Unit
IEC: Ion exchange chromatography
IF: Intestinal failure
ISTD: Internal Standard
ITx/IT: Intestinal transplantation
IV: Inverse variance
k_e: Elimination rate constant
LC/MS: Liquid chromatography-mass spectrometry
MANOVA: Multivariate analysis of variance
MD: Mean difference
MOOSE: Meta-analysis of Observational Studies in Epidemiology
MS or MS/MS: Mass spectrometer/mass spectrometry
MS: Mean Square
NEC: Necrotising enterocolitis
nm: nanometre
NO: Nitric oxide
Abbreviations

NOS: Nitric oxide synthase
OAT: Ornithine aminotransferase
OCT: Ornithine carbamoyltransferase
ODC: Ornithine decarboxylase
ORN/Orn: Ornithine
P/p: p-value
P5C: Pyrroline-5-carboxylate
P5CS: Pyrroline-5-carboxylate synthase
PN: Parenteral nutrition
PPi: Pyrophosphate
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses
psi: Pounds per square inch
QoL: Quality of life
ROC: Receiver operating curve
RTI: Research Triangle Institute
SBS: Short bowel syndrome
SBSQoLTM: Short bowel syndrome quality of life scale
SBTx: Small bowel transplantation
SD: Standard Deviation
SE: Standard Error
SENS: Sensitivity
SF-36: Short Form 36 questionnaire
SMD: Standardised mean difference
SPEC: Specificity
sROC: Summary receiver operating curve
SS: Sum of squares
SV: Screening visit
$\text{t}_{1/2}$: Half life
$T_{\text{max}}$: Time until maximum concentration
TMS: Tandem mass spectrometry
TN: True negative
TOMC: Time point of maximum concentration
TP: True positive
Abbreviations

TPN: Total parenteral nutrition
UC: Ulcerative colitis
UCH: University College Hospital
UCL: University College London
UCLH: University College London Hospitals
UPLC: Ultra Performance Liquid Chromatography
$V_d$: Apparent distribution volume
WMD: Weighted mean difference
$\Delta C_{\text{max}}$: Difference in $C_{\text{max}}$
$\mu$: micro
$\mu\text{M}$: $\mu\text{mol/L}$
Preface and Overview

Citrulline is a non-protein amino acid produced predominantly by the enterocytes of the intestine. This unique site of production has urged many researchers to examine whether citrulline plasma levels can operate as a reliable marker of enterocyte mass and functionality in conditions of diseased or resected intestine (short bowel syndrome, Crohn’s disease, coeliac disease etc.). In this thesis, various aspects of citrulline in relation to the intestine are investigated. The main questions of the thesis are:

1. Is citrulline being used in clinical practice? If so, then how is it being used (e.g. diagnostically, therapeutically, which particular conditions etc.)?

2. How is citrulline related to the intestine?

3. How can existing knowledge provide grounds to utilize citrulline as a marker to reflect intestinal function (absorption and possibly others)?

4. Can citrulline be a surrogate marker of other consequences related to bowel disease?

The thesis is organised around answering these questions. In Chapter 1, original historical details on the history of citrulline are presented, showing that citrulline was first isolated in 1914, contrary to the belief of 1930. The current “citrulline” is product of the watermelon (C. vulgaris) and is different from a previous “citrulline” which was a product of the bitter apple (C. colocynthis) and was used as a laxative. Next, a brief description of the biochemistry of citrulline is given alongside current measurement techniques. The knowledge about citrulline’s biochemical pathways is a result of quite extensive animal studies which have examined citrulline metabolism in developmental biology settings, in experimental medical conditions (massive small bowel resection, transplantation, mucositis, and internal anal sphincter function) and in physiological conditions. Overall, citrulline is part of pathways implicating glutamine and arginine metabolism as well and participates in two important cycles: the urea cycle and arginine-nitric oxide cycle. Important enzymes are ornithine aminotransferase, ornithine carbamoyltransferase, arginase, argininosuccinate synthase, argininosuccinate lyase, and carbamoyl phosphate synthase. A systematic review of animal studies was performed in Chapter 1 and suggests three large fields of citrulline research: biochemistry and developmental
biography of citrulline, clinical significance of citrulline, and citrulline function in colonocytes.

In Chapter 2, I focus my attention on the role of citrulline as a diagnostic marker in human clinical conditions. A systematic review of studies was performed along with a statistical meta-analysis. Overall, plasma citrulline measurements have been used in necrotizing enterocolitis, intestinal transplantation, short bowel syndrome, enteropathies (villous atrophy syndromes, Crohn’s disease, and mucositis) and critical illness patients. Citrulline was strongly positively correlated with enterocyte mass. This correlation was clinically significant in short bowel syndrome. In other conditions where short bowel isn’t an issue, there is a decrease in average citrulline levels compared to healthy controls and citrulline decrease can be correlated to the degree of disease severity apart from Crohn’s disease. Its interpretation should be cautious because its diagnostic accuracy is satisfactory but doesn’t exclude absolutely false negative or false positive cases. Factors that influence interpretation are lower population values, combination of intestinal disorders, possible bowel adaptation, measurement timing, inflammation and extent of disease. Citrulline was correlated positively with enteral absorption, albeit moderately. Although a higher plasma citrulline level suggests a more absorptive gut, this interpretation needs to take into consideration other factors such specific macronutrients, condition examined, extent of mucosal disease etc. A cut-off citrulline level at 20 μmol/L has sensitivity and specificity of 80% in all conditions. Limitations of the meta-analysis were presence of heterogeneity and the possibility of publication bias, detection bias and confounding bias.

In Chapter 3, I describe the design and results of the clinical study examining bioavailability of amino acids in healthy volunteers with a view to examine the potential utility of an oral citrulline stimulation test. The five oral challenges were the amino acids citrulline, glutamine and arginine against controls dextrose and 3-methylhistidine in a 5 × 5 crossover design (balanced Williams design). Post-absorptive amino acid levels of citrulline, glutamine, arginine, ornithine and 3-methylhistidine were measured with mass spectrometry and analysed with a crossover ANOVA and bioavailability testing. Overall, citrulline can function successfully as an oral challenge test. This oral challenge test is time dependent with citrulline decreasing after the oral challenge is performed. Interestingly, citrulline was the most potent stimulator for all other amino acids, despite glutamine
challenges being readily considered the most potent stimulators of citrulline production (Peters et al., 2007c; Peters et al., 2008b). This possibly demonstrates more efficient amino acid absorption and that this could be an important step to countering disease-related catabolism or assist in protein anabolism. Hence, citrulline challenges could be useful in intestinal failure but also in liver failure where urea cycle pathways including glutamine, arginine and ornithine are implicated. There were differences in the bioavailability of all five amino acids as shown with statistical testing (ANOVA \( p < 0.05 \) for citrulline, arginine and ornithine). There was no period, carryover or sequence effect except for 3-methylhistidine which had a significant period effect (possibly due to small concentrations). No treatment was equivalent to treatment with citrulline for producing equivalent post-absorptive concentrations \( (p > 0.05) \). Results were satisfactory from robustness, despite an apparent variability. Elements that provide robustness are powerful enough design with no carryover effects, acceptable accurate standard curves when analysing data in mass spectrometer, crossover ANOVA is reportedly robust and outlier analysis depicted markedly less influential points in the placebo treatment group in all plasma amino acids measured, except for plasma glutamine.

In Chapter 4, I present results regarding quality of life in patients with short bowel syndrome. This study was an investigation of quality of life in short bowel syndrome patients. The SBSQoL™ scale used is highly reliable in measuring quality of life in short bowel syndrome patients with a high Cronbach’s alpha (over 0.700). Four factors were extracted from the 17-item scale: general physical symptoms and activities, daily activities, digestive system symptoms, and stoma related symptoms. The main causes of low quality of life were fatigue, diarrhoea/increased stomal output, lack of sleep, gastrointestinal symptoms, and muscle pains. Lack of sleep has been frequently associated with short bowel syndrome predominantly due to long parenteral fluid infusion times and/or a daily infusion schedule because of large-volume fluid requirements, due to pumps and equipment alarms, fear of catheter dislodgement, nocturia, polyuria, need for extra storage space, and complaints of carrying heavy backpacks (Winkler and Smith, 2014). Physicians should treat these symptoms as much as possible to ease the burden of patients’ disease (e.g. loperamide for diarrhoea, paracetamol or stronger pain killers, zopiclone for sleeping). Due to the transitive property of associations,
the findings from this quality of life study suggest that changes of plasma citrulline levels are associated with changes with quality of life in patients with short bowel syndrome, due to the simultaneous association of quality of life and changes in citrulline levels with short bowel syndrome.

Finally in Chapter 5, I describe a summary of the thesis’ new findings, along with limitations and future work that could be performed based on this thesis’ results. The present thesis creates the potential for future studies in the field. These could involve:

1. A dynamic study with oral citrulline challenges in states of compromised intestine, such as extensive enteropathies (e.g. coeliac disease, mucositis, radiation, Crohn’s disease) or short bowel syndrome or intestinal failure.
2. Citrulline challenges in liver failure, since citrulline is part of the urea cycle.
3. Oral challenges in cases of compromised gut during critical illness. This is similar to extensive enteropathy but it is broader since multiple factors can contribute to critical illness enteropathy: endotoxemia, sepsis, catabolism, nitric oxide changes, arginine fluxes, presence of excessive lactate, etc. In Chapter 2 it was shown that citrulline was measured in critical illness but mainly as a static measure, with all the relevant shortcomings. A study in this setting would examine the potential of an oral challenge against single or serial measurements of citrulline.
4. A larger study for quality of life examining quality of life in conjunction with other psychosocial parameters, other quality of life scales, plasma citrulline levels, test re-test reliability, construct validity and responsiveness. Quality of life studies should be able to differentiate quality of life affected by home parenteral nutrition and short bowel syndrome.
Chapter 1
Introduction

Citrulline, a non-protein amino acid, has attracted research groups’ interest around the world more predominantly in the last decade. What are those features that make citrulline so special? Why are researchers investigating this amino acid with profound interest? How widespread is citrulline measurement among researchers and practitioners and what findings have been filling the pages of scientific journals regarding citrulline?

These are certain aspects that Chapter 1 of the thesis will try to answer. However, it will take a focused view of citrulline with respect to its role in intestinal physiology and pathology. It will critically review the literature and try to synthesize statistically the literature, where possible, to provide significant outcomes to discuss. Hence, this chapter will first discuss the history of citrulline, then move on to the metabolism of citrulline, important biochemical pathways and measurement methods for citrulline; and finally a systematic review of citrulline and the intestine in animal studies.

1.1 History of Citrulline

“Citrulline was first isolated in 1930” (Moinard et al., 2008). This is a common phrase appearing when we read nutritional and gastroenterology literature regarding citrulline. Nevertheless, some interesting historical sources detail that the amino acid citrulline was discovered much earlier, while there was use of another “citrulline” in the late 19th century.

To begin with, in the North Carolina Medical Journal, January 1883 (1883a, p. 44), the following passage can be read:

The colocynthum purum prepared by Merck […] produces watery stools with moderate torments […] There is also a resinoid substance called citrullin, extracted from the colocynth fruit, insoluble in water, which, when taken internally in the dose of 5 milligrammes to 1 centigramme, or if administered hypodermically in the same dose […] will produce the desired effect.

---

1 Acute abdominal pain
Similar excerpts appeared then in other journals, such as the *Medical Age* (1894) (Figure 1.1) and the *Journal of the American Medical Association* (1909), and a significant gastroenterologist of that time, Ismar Boas (1858-1938) (Hoenig and Boyle, 1988), comments on citrulline’s usefulness with difficult cases of constipation (Boas, 1904). To the reader of nutrition and gastroenterological articles, this early mentioning of citrulline causes some surprise. That is because citrulline is generally regarded as having been first discovered in the 1930s (Wada, 1930a) and found to play a key role in the urea cycle. A therapeutic action is not generally attributed to citrulline, so this laxative effect also surprises us. Below I explore the history of the citrullines since, as will become clear, there is more than one.

### 1.1.1 Early Sources Mentioning the Term “Citrulline”

I performed a systematic review of all readily available texts dating before 1930, which mentioned the term “citrulline”. A preliminary analysis had shown us that the synonymic terms “citrullin” and “citrullinum” were also in use. I searched databases with extensive digitization of volumes of rare books including Google Books ([http://books.google.com](http://books.google.com)), Internet Archive ([http://www.archive.org](http://www.archive.org)), and Gallica ([http://gallica.bnf.fr](http://gallica.bnf.fr)). These three databases are the largest online sources of mass digitization projects of books from a large range of American and British universities, and of the National Library of France. The search terms “citrulline”, “citrulline” and “citrullinum” for the period 1800-1930. A source of bias might have been introduced, due to the fact that languages which do not use the Latin alphabet were not searched.

Results revealed that 170 books and journal volumes have the word citrulline among their pages. These were all published between 1882 and 1930 and are mainly English, French, and German texts, with very few in Dutch, Swedish, and Italian (Appendix A). Almost all texts mention the results of two main articles, written by Hiller (1882) and Kohlstock (1892), discussing subcutaneous and rectal injection of purgatives.

This citrulline is a resin produced by the pulp of *Citrullus colocynthis*. *C. colocynthis* Schrad (family Cucurbitaceae), also known as colocynth or bitter apple, which is a common weed found in countries of the Middle East and the Mediterranean (Figure 1.2). *C. colocynthis* has been used medicinally since ancient
times (Hatam et al., 1989). The fruits and seeds are used as a purgative and have been suggested to possess antitumor activity (Chaturvedi et al., 2003). From the pulp of C. colocynthis, α-elaterin, α-elaterin-2-D-glucopyranoside, citrullol, and an alkaloid with strong purgative action have been isolated (Power and Moore, 1910), while the other parts of this weed contain several more potentially active substances (El Khadem and Abdel Rahman, 1963; Hatam et al., 1989, 1990). When overconsumed, it can cause colitis (Jansen, 1889; Goldfain et al., 1989).

This nineteenth century citrulline is a strong purgative used occasionally in humans. In two articles dealing with subcutaneous and rectal injection of purgatives (Hiller, 1882; Kohlstock, 1892), both authors found that citrulline could be administered subcutaneously or rectally (dose: 5 mg to 20 mg) in cases of chronic constipation, but it was followed by adverse effects of severe pain, oedema and redness of the skin. Rectal injections were tolerated more easily than subcutaneous ones. Its use was most possibly hindered because of a high price or low availability (Bandler, 1915), while it was used more frequently in veterinary medicine (1907). It appears to have passed into oblivion over time possibly reflecting progressively infrequent use of a substance with a poor risk-benefit ratio or a change in its name, making it difficult to track in scientific literature.

1.1.2 The History of Modern Citrulline’s Isolation

Modern citrulline is a non-protein amino acid, and in humans its plasma content is derived largely from the amount produced in enterocytes of the small bowel (Windmueller and Spaeth, 1981) (Figure 1.3). Citrulline’s first isolation from the juice of the watermelon (Citrullus vulgaris Schrad) has generally been attributed to Mitsunori Wada (Kornberg, 2000; Curis et al., 2005; Mandel et al., 2005; Moinard and Cynober, 2007; Moinard et al., 2008), who isolated citrulline and determined its chemical formula in 1930, naming the substance he isolated citrulline (Wada, 1930a). In fact, it was isolated much earlier.

The first isolation of citrulline in 1914 was by Yotaro Koga and Ryo Ohtake (Koga and Ohtake, 1914). They isolated a substance from the juice of the watermelon with the chemical formula C$_6$H$_{13}$N$_3$O$_3$ (Figure 1.4 and Appendix B). They did not further elucidate the structure of this new substance nor did they name it. A possible brake on wider dissemination of this new understanding was the fact that the article was
written in Japanese, making it difficult for European and American scientists to grasp its significance.

In 1930, Wada (1930a) was therefore repeating the Koga-Ohtake experiment in the same laboratory (Agricultural Chemical Laboratory, Tokyo Imperial University). He went on to define its chemical formula and structure and to prove that his observations were correct by synthesizing the new amino acid. He named this “citrulline” (Wada, 1930a, b). He further demonstrated its isolation from the tryptic digestion of casein and possibly arginine (Wada, 1933). He published his findings in the German journal *Biochemische Zeitschrift* (Wada, 1930a) and in the *Proceedings of the Imperial Academy* in English (Wada, 1930b). Publishing in German and English was of great significance since it enhanced the swift acceptance of his results.

The first isolation by Koga and Ohtake (1914) was however acknowledged by other researchers over the following years. Koga and Ohtake (1914) were attributed with its isolation from the juice of watermelon, and Wada (1930a) with the first sound study determining the chemical properties and structure of citrulline, as well as coining its name (Wada, 1930a; Vickery, 1941; Kornberg, 2000). Wada 1930a, p. 420) himself acknowledges this contribution to the first isolation of citrulline.

“Sixteen years ago in this laboratory, Y. Koga and S. Odake isolated from the juice of watermelon a nitrogenous compound as colorless prisms, different from arginine and Glycocoll betaine… The analysis yielded the empirical formula C₆H₁₃N₃O₃. Since then, the structure has not been elucidated”.

In 1931, Ackermann (1931, p. 66) indicates that “such a substance had already been isolated from the watermelon, Citrullus vulgaris, by Y. Koga and S. Odake, and its structure had been fully described by Wada”. In 1941, Vickery (1941, p. 95) records “In 1914, Koga and Odake described the isolation of a substance C₆H₁₃N₃O₃ from the juice of watermelon. Aside from the fact that it formed a copper salt, little else was recorded. In 1930, Wada prepared the substance again and showed that its properties were best explained on the assumption that it is L-carbamido ornithine”. This account is repeated in a later review on the discovery of amino acids (Vickery, 1972).

---

*Wada (1930b) possibly spells Ryo Ohtake’s surname as Odake. This spelling is used by all the other authors citing their work after 1930. We have kept the original spelling, according to our translation, but Ohtake and Odake refer to the same individual.*
Krebs and Henseleit (1932) demonstrated that citrulline is an intermediate in the mechanism whereby urea is formed in the liver, signifying the importance of this amino acid in nitrogen metabolism (Fearon, 1939), citing first the work by Koga and Ohtake (1914), and then that of Wada (1930a). Fearon (1939, p. 902) notes that “the carbamido-acid citrulline, isolated by Koga & Odake in 1914 from the watermelon, attracted no general attention until Wada established its constitution as α-amino-δ-carbamidovaleric acid”. Finally, Impellizzeri et al. (1975), while claiming that citrulline has been detected in a variety of plant sources and presumably is universal in plants (because of its role in arginine biosynthesis), cite the work by Koga and Ohtake (1914) as the first to have isolated citrulline from the juice of watermelon.

In conclusion, before 1930, when modern citrulline was first believed to be isolated, there was another use of the term citrulline, signifying a resin produced by C. colocynthis. This citrulline is different from modern citrulline. In addition to this, modern citrulline was not isolated in 1930 but surprisingly somewhat earlier. Reviewing the original manuscript indicates that Koga and Ohtake (1914) isolated citrulline for the first time in 1914, even though their work did not lead to the determination of its structure and nature.

1.2 Biochemical and Analytical Properties of Citrulline

Until recently, citrulline had not managed to attract the nutrition community’s interest, most probably because citrulline is a non-protein amino acid, and was viewed solely as a metabolic intermediary in the urea cycle. Also, citrulline is almost absent from natural foods, with the watermelon being a notable exception (Curis et al., 2005; Cynober et al., 2010).

1.2.1 Citrulline Metabolism

Citrulline is a colourless solid at ambient temperature and pressure. Its melting point is 222°C. It is an α-amino acid with an asymmetric carbon; hence, it presents two enantiomers. Like most amino acids, its natural form is the L form.

Citrulline is derived from arginine, either directly via a reaction mediated by nitric oxide synthases or indirectly via conversion by arginase into ornithine, which in turn is acted on by ornithine carbamoyltransferase to deliver citrulline. Another
indirect citrulline source is glutamine, via a series of reactions illustrated in Figure 1.5.

Citrulline is metabolized by a unique reaction, catalysed by argininosuccinate synthase, into argininosuccinate, which is immediately converted back to arginine by argininosuccinate lyase (Figure 1.5). Arginase, ornithine carbamoyltransferase, argininosuccinate synthase and argininosuccinate lyase are all expressed simultaneously only in periportal hepatocytes in the Krebs-Henseleit cycle, also known as the urea cycle (Krebs and Henseleit, 1932). Other cells expressing some of these enzymes enable intra- or extra-cellular cycles, as described in Table 1.1, with the kidneys playing the most important function in the degradation of citrulline. These cycles are of physiological significance; for example, up to 20% of nitric oxide production is derived from recycled arginine, while in patients with severe kidney problems such as renal failure, high citrulline plasma levels is a frequent finding (Rabier and Kamoun, 1995; Curis et al., 2005).

1.2.2  Intracellular Cycles

Citrulline recycling into arginine is particularly efficient in macrophages, making citrulline almost as efficient as arginine at producing nitric oxide, especially against a background of low arginine availability such as in inflammatory states. Macrophages not only express both argininosuccinate synthase/argininosuccinate lyase and nitric oxide synthase but also arginase type II and ornithine decarboxylase. Thus macrophages can synthesize nitric oxide or aliphatic polyamines.

1.2.3  Inter-organ Cycle

The inter-organ exchanges of citrulline are summarized in Figure 1.6. The citrulline interorgan cycle was first described by Windmueller and Spaeth (1981), who established that citrulline was synthesized in the intestine from glutamine and arginine. Later on, a close correlation between glutamine uptake and citrulline release by the small intestine was demonstrated in adults (Crenn et al., 2008). In new-borns, the gut releases arginine and not citrulline, due to high argininosuccinate synthase/argininosuccinate lyase expression. The reason for this is that breast milk is not sufficiently rich in arginine to meet requirements, coupled with the fact that the kidney is not mature and its arginine production is still low (Köhler et al., 2008). A feature of this inter-organ cycle is the absence of any
significant hepatic uptake or release of citrulline, hepatic citrulline metabolism being strictly compartmentalized to periportal hepatocytes (Rabier and Kamoun, 1995).

This inter-organ cycle plays a key role in the modulation of nitrogen homeostasis according to dietary protein availability (Curis et al., 2005). After a protein meal, the urea cycle is activated by high arginine availability, since arginine is a potent inducer of ureagenesis, both as a cycle intermediary and as an activator of N-acetylglutamate synthase (Figure 1.5). N-acetylglutamate is the allosteric activator of carbamoyl-phosphate synthase. In other words, ureagenesis is activated as more arginine appears in the portal blood. The only other amino acid able to produce citrulline in the gut is glutamine, which is also the only other amino acid able to strongly activate ureagenesis.

Ultimately, arginine in the splanchnic area functions as a sensor of excessive nitrogen intake, since high arginine in the portal blood reflects high protein intake: activation of ureagenesis in this condition prevents excessive, potentially neurotoxic amino acid levels in the general circulation, and provides a way to decrease arginine availability in the general circulation and thereby avoid excessive nitric oxide production.

When protein (i.e. arginine and glutamine) availability is low, derepression of ornithine carbamoyltransferase in the enterocyte leads to release of citrulline (instead of arginine) by the intestine. Since citrulline bypasses the liver, this slows down the urea cycle and avoids nitrogen wastage, as arginine requirements are covered by resynthesis in the kidney. In other words, citrulline is a masked form of arginine that bypasses the liver (arginine synthesized from citrulline represents 60% of the de novo synthesis of arginine in adults).

1.2.4 Analytical Methods to Quantify Citrulline

Various methods can be used to quantify citrulline, either specifically or similar to other amino acids (Curis et al., 2007). Only the most frequently used and recently proposed methods are discussed here.
1.2.4.1 Ion Exchange Chromatography

Ion exchange chromatography with post column derivatization (ninhydrin) is the reference method for amino acid analysis. The dosage is fully automatized and the performance (between-run reproducibility) for citrulline measurement is good, with a coefficient of variation of 4.3 % (Neveux et al., 2003). A short program allows specific measurement of citrulline in 30 minutes from run-to-run. The limitation of this method is its relatively low sensitivity, making it inadequate for arteriovenous difference measurement.

1.2.4.2 Reversed Liquid Phase Chromatography

Citrulline concentration can be measured by reversed liquid phase chromatography using various precolumn derivatization agents, in particular o-phtalaldehyde. The between-run reproducibility is not as good as for ion exchange chromatography but the dosage is more sensitive, allowing measurements of arteriovenous differences (Curis et al., 2005).

1.2.4.3 Mass Spectrometry on Dried Blood Spot

Citrulline has recently been measured by hydrophilic interaction chromatography/mass spectrometry/mass spectrometry on dried blood spot specimens to monitor graft function following intestinal transplantation. The advantages of this method are that it is almost non-invasive and requires minimal blood sampling (< 25 µL). Also, hydrophilic interaction chromatography/mass spectrometry/mass spectrometry detection has high sensitivity and a low limit of detection (1.5 µmol/L). Using dry blood spot specimens induces large variations compared to results obtained using plasma, however. For example, patients with a citrulline concentration of 60 µmol/L using the dry blood spot method may have a plasma concentration ranging from 38-90 µmol/L. Similar uncertainty is found for low values (dry blood spot measured at 15 µmol/L equals plasma citrulline levels between 10 µmol/L and 40 µmol/L) (Yu et al., 2005; Englund et al., 2014).

1.2.4.4 Ultra Performance Liquid Chromatography Coupled with Mass Spectrometry

Ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) is performed with a SystemSolvent delivery and sample introduction is
performed using a Waters ACQUITY Ultra Performance Liquid Chromatography system (Waters, Milford, MA, USA) equipped with a thermostat for both the sample and column compartments which are maintained at 4°C and 55°C, respectively. A Waters ACQUITY TQ tandem quadrupole mass spectrometer, interfaced with an atmospheric pressure electrospray ionization (AP-ESI) source is used for the analysis. Separation is performed on a Hydrophilic Interaction Liquid Chromatography (HILIC) column, 2.1 mm × 100 mm, packed with 1.7 μm particles designed to withstand 15,000 psi. Mobile phases A and B consist of distilled water containing 0.1% (v/v) formic acid, and acetonitrile with 0.1% formic acid, respectively. The gradient program is as follows: flow rate in all steps at 0.45 mL/minute unless otherwise indicated. Initial: 90% B; 0-2.5 minutes: a gradient to 55% B; 2.5-3.5 minutes: 2% B; 3.5-4.9 minutes: reversion of the mobile phase to 90% B at a flow rate of 0.60 mL/minute; 4.9-5 minutes: 90% phase B. Full loop injection is used to introduce 10 μL of sample into the system.

The atmospheric pressure electrospray ionization is operated in the positive ion mode. Nitrogen is used as the nebulizing and desolvation gas at a flow rate of 100 and 1000 L/hour, respectively; argon at a pressure around 3 ×10⁻³ mbar will be used as collision gas at a flow rate of 15 mL/hour. The ion source and the desolvation temperature is maintained at 130°C and 400°C, respectively. All aspects of system operation and data acquisition is controlled using Masslynx version 4.1 software with automated data processing using the Quanlynx Application Manager (Waters) (Demacker et al., 2009a; Demacker et al., 2009b).

This section was a general introduction to the biochemistry of citrulline in the body and methods for analytical measurement of citrulline. In the next section, a systematic review of the available literature on citrulline is presented and possible theories that explain the findings are presented.

### 1.3 Non-Human Studies of Citrulline and the Intestine

For this section, a systematic review of the literature was done. The inclusion criteria for this systematic review were: any empirical study (abstract and full paper) describing investigation of citrulline in relation to the term intestinal function in animals. The only papers that were excluded were those whose object of investigation was not related to intestinal function. There was no restriction to
language of papers and the type of studies. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for systematic reviews were used (Moher et al., 2009).

Electronic database searches were conducted in Google Scholar, Pubmed/Medline, Scopus, and EMBASE with no year limits. Publisher databases were also searched (sciencedirect.com, link.springer.com, Wiley Library Online, Highwire Press, nature.com, Ovid, Cambridge University Press). The search keywords were: citrulline, intestine, gut, non-human populations, mucositis, resection, transplant, radiotherapy, cancer, enterocyte, and colitis. The date of search was up until 1 July 2015. The bibliographies from all included manuscripts and hand searching of relevant gastroenterology and nutrition journals were used to identify further references. The resulting studies (in abstract form) were assessed against the inclusion criteria. When there was insufficient information available in the abstract, the full text was reviewed. Then, data were extracted from the selected studies including: author, year of publication, aim of the study, study design, and results.

The search yielded 226 studies, out of which 80 involved some study protocol examining citrulline metabolism in relation to the intestine or intestinal function in animals other than humans (Figure 1.7). The design and basic findings of all papers are shown in Tables 1.2 and 1.3.

The period of citrulline animal studies begins in the 1980s, when the papers by Windmueller and Spaeth (1981) and others pointed out that plasma citrulline is produced predominantly by the intestine and described basic biochemical pathways. The second period begins in the early mid-1990s with the studies by Wu et al. (1994a); Wu et al. (1994b), who produced very important clarifications in the pathways of citrulline production and the enzyme activity during initial postnatal stages. Finally, the third period of studies involves the 2000s, particularly after the seminal papers by Crenn et al. (2000); Crenn et al. (2003) in the significance of citrulline levels for predicting various grades of intestinal failure. The animal models used involve pigs, mice, rats and sheep. The studies can be categorised into the following topics, discussed below.
1.3.1 Biochemistry and Developmental Biology of Citrulline

The majority of non-human studies involve biochemistry, which deal with biochemical pathways in cells, molecules and interorgan crosstalks, and developmental biology, which studies the process by which organisms grow and develop. The basic metabolism of citrulline was described in the previous chapter, but this knowledge was gradually built upon the published results and studies confirming them. Windmueller and Spaeth (1981) described in a model of rats that circulating citrulline was the result of production in the enterocyte; the chemical pathway leading to citrulline’s production was also clarified, involving the enzymes ornithine aminotransferase, ornithine carbamoyltransferase, pyrroline-5-carboxylate synthase, and nitric oxide synthase among others, with mainly the amino acids glutamine, ornithine and arginine affecting the production of citrulline. The full classification of papers according to categories is shown in Tables 1.2 and 1.3.

Some interesting papers are described. Recently, a chemical kinetics paper by Bensaci et al. (2012) point out that in physiological and pathological conditions of the enterocyte, the most important enzyme affecting citrulline was ornithine aminotransferase with similar results originally described in the past as well (Wu, 1997; Wu et al., 1997; Dekaney et al., 2001, 2003). It is commonly accepted that arginine increases citrulline concentrations through the nitric oxide synthase pathway, with many descriptions going as back as 1976 (Herzfeld and Raper, 1976; Uchiyama et al., 1981; Hurwitz and Kretchmer, 1986; Edmonds et al., 1987). However, a recent study in a rat model of ischaemia and reperfusion injury showed that administering arginine decreased citrulline; this reduction in levels was attributed to the fact that during long term arginine administration, arginine may not be metabolized efficiently in late post-injury stages (Lee et al., 2012).

In parallel with developments in biochemistry, progress has been made in the developmental biology of citrulline and its synthesizing enzymes. There has been a noted difference in citrulline’s chemical pathways enzymes during perinatal periods in pigs and chicks as well as before and after weaning. In perinatal mice (Hurwitz and Kretchmer, 1986; de Jonge et al., 1998) and piglets (Wu et al., 1994b; Wu et al., 1995a; Wu et al., 1995b; Wu et al., 1996; Wu et al., 1997), all enzymes necessary for arginine biosynthesis from proline and glutamine, are expressed in the enterocytes of the small intestine, while arginase, the main cytosolic arginine-
catabolizing enzyme, is not detectable prior to weaning (Wu et al., 1995a). In agreement with this, the small intestine plays a prominent role in net arginine production in suckling piglets (Wu et al., 1994a; Wu et al., 1994b; Urschel et al., 2005; Urschel et al., 2006). In rodents, intestinal expression of the enzymes that synthesize arginine from citrulline, argininosuccinate synthetase and argininosuccinate lyase, ceases completely after weaning (de Jonge et al., 1998). In pigs, on the other hand, net synthesis of arginine declines more gradually and is still present at seven weeks of age (Wu, 1997). It has been suggested that enteric arginine synthesis is necessary to cover neonatal requirements, because mammalian milk is a relatively poor source of arginine, whereas its precursors proline and glutamine are abundant (Köhler et al., 2008).

1.3.2 Clinical Significance of Citrulline

Three major themes dominate this field: citrulline levels, metabolism and therapeutic effects in mucositis, intestinal resection and transplantation. There have also been studies in cancer models, endotoxemia and necrotizing enterocolitis. For radiation induced mucositis, there is a universal consensus that citrulline markers correlate with intestinal function markers such as villous depth or villous morphology (Lutgens et al., 2003; Siqueira et al., 2010; Fijlstra et al., 2011). For transplantation, citrulline was examined in pigs as a marker for graft rejection. Plasma citrulline levels failed as a marker in the early diagnosis of acute cellular rejection and became reliable only when advanced mucosal damage was present (Nadalin et al., 2007). Finally, in intestinal resection citrulline decreases massively and arginine becomes an essential amino acid which supplements nitrogen (Wakabayashi et al., 1994; Wakabayashi et al., 1995; Lardy et al., 2004; Osowska et al., 2004; Osowska et al., 2006; Osowska et al., 2008). The other studies on necrotizing enterocolitis, endotoxemia and cancer assess citrulline levels as surrogate markers of intestinal function examining it in conjunction with their other primary objectives.

1.3.3 Citrulline Function in Colonocytes

Citrulline recycling may be responsible for the maintenance of internal anal sphincter relaxation during nonadrenergic, noncholinergic nerve stimulation. Also citrulline has been studied in the context of nitric oxide inhibitors and their use in
colonocytes (Grider and Jin, 1993; Berrino et al., 1995; Shuttleworth et al., 1995; Shuttleworth and Sanders, 1996; Chakder and Rattan, 1997; Rattan and Chakder, 1997; Shuttleworth et al., 1997; Armstrong et al., 2000).

1.4 Summary and Conclusion

Original historical details on the history of citrulline were presented, showing that citrulline was first isolated in 1914, contrary to the belief of 1930. The current “citrulline” is product of the watermelon (C. vulgaris) and is different from another “citrulline” of the past which was product of the bitter apple (C. colocynthis) and was used as a laxative. Next, a brief description of the biochemistry of citrulline was given alongside current measurement techniques. The knowledge about citrulline’s biochemical pathways is a result of quite extensive animal studies which have examined citrulline metabolism in developmental biology settings, in experimental medical conditions (massive small bowel resection, transplantations, mucositis, and internal anal sphincter function) and in physiological conditions. Overall citrulline is a result of glutamine and arginine metabolism and participates in two important cycles: the urea cycle and arginine-nitric oxide cycle. Important enzymes are ornithine aminotransferase, ornithine carbamoyltransferase, arginase, argininosuccinate synthase and lyase and carbamoyl phosphate synthase. A systematic review of animal studies was performed and suggests three large fields of research: biochemistry and developmental biology of citrulline, clinical significance of citrulline, citrulline function in colonocytes. Overall, this information suggests that there is a potential for citrulline (diagnostically or therapeutically) for use in clinical practice and this will be focus of Chapter 2, where a systematic review and meta-analysis of human studies examining citrulline’s diagnostic role are presented.
### 1.5 Tables

**Table 1.1.** Sites and behaviour of citrulline catabolism into arginine through argininosuccinate synthase/argininosuccinate lyase (Breuillard *et al.*, 2015; Wijnands *et al.*, 2015).

<table>
<thead>
<tr>
<th>Organ or cells</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Arginine release to meet tissue requirements</td>
</tr>
<tr>
<td>Immune cells</td>
<td>Recycling into arginine for nitric oxide synthesis or polyamine synthesis (mainly in macrophages)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Recycling into arginine for nitric oxide synthesis</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Fuels arginine in the urea cycle</td>
</tr>
<tr>
<td>Gut</td>
<td>Only in new-borns (downregulation of argininosuccinate synthase/argininosuccinate lyase activities at weaning); arginine production to meet requirements (mostly because arginine from the milk fails to do so)</td>
</tr>
</tbody>
</table>
Table 1.2  Animal or cellular studies investigating citrulline in the intestine.

<table>
<thead>
<tr>
<th>No.</th>
<th>Authors</th>
<th>Settings</th>
<th>Findings</th>
<th>Categorization</th>
</tr>
</thead>
</table>
| 1   | Beaufrere et al. (2014) | - Aim: to investigate whether the effect of glutamine on the increase in intestinal villus height is correlated with an increase in both gut mass and citrulline plasma level in very old rats.  
- Design: Very old (27 months) female rats were supplemented with oral glutamine (20% of diet protein).  
- Intestinal histomorphometric analysis of the small bowel was performed.  
- Amino acids, in particular citrulline, were measured in the plasma, liver and jejunum.                                                                 | - Total intestine mass was significantly higher in glutamine-supplemented rats than in controls (15%).  
- Measurement of villus height and crypt depth demonstrated that the difference between villus and crypt was significantly improved in glutamine pre-treated rats compared to controls (~ 11%).  
- Plasma citrulline also increased by 15% in glutamine-supplemented rats compared to controls. | Clinical Significance (Intestinal Mass)                                                                                      |
| 2   | Gutierrez et al. (2014) | - Aim: this study compared serial serum citrulline and mucosal adaptive potential after proximal versus distal small bowel resection.  
- Design: Enterally fed Sprague-Dawley rats underwent sham operation or 50% small bowel resection, either proximal or distal.  
- Citrulline was measured at operation and weekly for 8 weeks.  
- At necropsy, histologic features reflecting bowel adaptation were evaluated.                                                                 | - By weeks 6-7, citrulline in both resection groups significantly decreased from baseline and was significantly lower than the concentration in sham animals.  
- There was no difference in citrulline between proximal resection and distal resection at any point. Villus height and crypt density were higher in the proximal resection than in the distal resection group. | Clinical Significance (Intestinal Resection)                                                                |
| 3   | Shim et al. (2014)     | - Aim: to establish an appropriate and efficient minipig model to study high-dose radiation-induced gastrointestinal syndrome after radiation exposure.  
- Design: Ileocutaneous anastomosis was performed 3 weeks before irradiation in six male Göttingen minipigs.  
- Minipigs were locally irradiated at the abdominal area using a gamma source as follows: 1,000 cGy (n = 3) and 1,500 cGy (n = 3).  
- Endoscopic evaluation for the terminal ileum was periodically performed via the ileocutaneous anastomosis tract. Pieces of tissue were serially taken for histological examination. | - Mucosal atrophy and telangiectasia was present in the ileum from day 1 to day 17 after abdominal irradiation.  
- Microscopic findings were characterized as architectural disorganization, loss of villi and chronic active inflammation. Increase in cyclooxygenase-2 expression was closely correlated with severity of tissue damage and inflammation.  
- Plasma citrulline level was significantly decreased the day after irradiation and recovered when irradiated mucosa was normalized.  
- Results showed that citrulline changes were positively correlated with microscopic changes and the endoscopic score in radiation-induced mucosal damage. | Clinical Significance (Intestinal Radiation Damage)                                                      |
| 4   | Cakmaz et al. (2013)   | - Aim: To measure the plasma levels of diamine oxidase and citrulline in acute mesenteric ischaemia to gain insight into its early diagnosis.  
- Design: 21 Wistar albino rats were divided into three groups, control group, short-term ischemia group, and prolonged ischemia group. The superior mesenteric artery was occluded for 15 minutes in the short-term ischemia group and for 12 hours in the prolonged ischemia group.  
- Twelve hours later, the experiment was terminated and plasma diamine oxidase and citrulline levels were measured. Intestinal tissue was evaluated for the histopathological changes. | - Compared to the control group, the short-term and prolonged ischemia groups showed significant increases in the plasma levels of diamine oxidase, whereas the plasma citrulline levels decreased significantly.  
- Prolonged ischemia caused a larger increase in the plasma diamine oxidase levels and a larger decrease in the plasma citrulline levels compared to the short-term ischemia (p = 0.011 and p = 0.021, respectively). | Clinical Significance (Ischaemia)                                                                       |
### Table 1.2 (Continued)

<table>
<thead>
<tr>
<th>No.</th>
<th>Authors</th>
<th>Settings</th>
<th>Findings</th>
<th>Categorization</th>
</tr>
</thead>
</table>
| 5   | Fijlstra et al. (2013)   | Aim: To compare the quantitative capacity to absorb glucose between rats with methotrexate-induced mucositis and saline-treated controls. | • Methotrexate-induced mucositis was confirmed by a reduction in villus length and plasma citrulline (both ∼57%, relative to controls, *p* < 0.01).  
• When glucose was administered as a bolus, methotrexate-treated rats only absorbed 15% of administered glucose, compared with 85% in controls (*p* < 0.01).  
• Upon continuous intraduodenal glucose infusion, the median absorptive capacity for glucose in methotrexate-treated rats did not differ from controls (80% versus 93% of administered glucose respectively, *p* = 0.06).  
• Glucose absorption differed substantially between individual methotrexate-treated rats (range, 21%–95%), which correlated poorly with villus length (*rho* = 0.54, *p* = 0.030) and plasma citrulline (*rho* = 0.56, *p* = 0.024). | Clinical Significance (Mucositis) |
| 6   | Nakamura et al. (2013)   | Aim: To investigate whether dietary glutamate-N is an effective nitrogen source for amino acid synthesis and investigated the fate of dietary glutamate-N using [15N]glutamate. | • Most of the dietary glutamate-N was metabolized into CO₂, lactate, or alanine (56%, 13%, and 12% of the dietary input, respectively) in the portal drained viscera.  
• Most of the glutamate-N was utilized for the synthesis of other amino acids such as alanine and citrulline (75% and 3% of dietary input, respectively) in the portal drained viscera, and only minor amounts were released into the portal vein in the form of ammonia and glutamate (2% and 3% of the dietary input, respectively). | Biochemistry                    |
| 7   | Marini et al. (2012)     | Aim: To test whether during the neonatal period de novo synthesis is the main source of ornithine for citrulline synthesis | • During fasting, plasma proline (13%) and ornithine (19%) were main precursors for citrulline synthesis, whereas plasma arginine (62%) was the main precursor for plasma ornithine.  
• During feeding, enteral (27%) and plasma (12%) proline were the main precursors for the ornithine utilized in the synthesis of citrulline, together with plasma ornithine (27%).  
• Enteral proline and glutamine were utilized directly by the gut to produce ornithine utilized for citrulline synthesis.  
• Arginine was not utilized by the gut, which is consistent with the lack of arginase activity in the neonate.  
• Arginine, however, was the main source (47%) of plasma ornithine and in this way contributed to citrulline synthesis. | Biochemistry                    |
| 8   | John-Baptiste et al. (2012) | Aim: To test selected candidate biomarkers with regards to their usefulness as gastric injury biomarkers in this study.  
• Design: Biomarkers included plasma diamine oxidase and citrulline, faecal calprotectin, bile acids, and miRNA.  
• p21-activated kinase 4 inhibitor as a preclinical rat model of gastric toxicity.  
• Wistar Han rats | • L-citrulline and miR-194 results appear to correlate well with histopathology findings.  
• The results for L-citrulline levels in rat plasma detected by mass spectrometry showed a significant dose- and time-dependent decrease with a ∼60% decrease on day 3 and ∼90% decrease on day 5.  
• The decrease on day 5 compared with baseline was consistent with an overall reduction in enterocyte mass, which manifested histologically as crypt necrosis and villus atrophy and fusion. | Clinical Significance (Mucositis) |
### Table 1.2 (Continued)

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| 9   | Batista *et al.* (2012) | - Aim: To assess citrulline effects on gut barrier integrity and bacterial translocation in mice undergoing intestinal obstruction  
- Design: Mice were divided into 3 groups: sham, intestinal obstruction, and citrulline. The citrulline group received a diet containing 0.6% citrulline; the intestinal obstruction and sham groups were fed a standard chow diet.  
- Terminal ileum was ligated except the sham group, which only underwent laparotomy. | - The citrulline group presented decreased intestinal permeability and bacterial translocation when compared with the intestinal obstruction group.  
- Histopathology showed that citrulline preserved the ileum mucosa  
- The intestinal secretary Immunoglobulin A concentration was higher in the citrulline group. The intestinal obstruction group presented the highest levels of interferon-γ. | Biochemistry  
Therapeutic significance of citrulline |
| 10  | Bensaci *et al.* (2012) | - Aim: To production of mathematical model of the enterocytic glutamine to citrulline conversion in the fasting state  
- Design: Two different approaches were compared: a standard approach based on the Michaelis-Menten assumptions (King-Altman approach) and an association-dissociation approach based on the kinetic mass action law (Van’t Hoff approach)  
- Cellular study | - In both cases, the model correctly predicts the physiological plasma citrulline steady-state, but the two approaches present clear differences for metabolites of enzymes having a complex mechanism, challenging the validity of the King-Altman approach in such cases.  
- When physiopathological scenarios of enzyme activity loss are simulated, both approaches predict a very sharp transition from the physiological citrulline plasma level to the lack of its production: the concentration profiles of these simulations show a clear threshold of which characteristics vary with the involved enzyme.  
- Ornithine aminotransferase shows the highest sensitivity in the system whatever the approach used. | Biochemistry |
| 11  | Boutry *et al.* (2012) | - Aim: to examine whether glutamate, glutamine and citrulline concentrations in blood, intestine and muscle are decreased by endotoxemia, and if supplementation with glutamate or glutamine can restore normal concentrations  
- Design: Endotoxemia induced in rats by an intraperitoneal injection of lipopolysaccharide. | - Endotoxemia rapidly but transiently decreased the circulating concentrations of almost all amino acids and more durably of glutamate, glutamine and citrulline in muscle.  
- Supplementation with glutamate or glutamine failed to restore glutamate, glutamine and citrulline concentrations in plasma and muscles. | Clinical Significance (Endotoxemia) |
| 12  | Chapman *et al.* (2012) | - Aim: to investigate whether arginine or its precursor, citrulline protects intestinal tight junctions from hypoxia and determined if inducible nitric oxide plays a role.  
- Design: Neonatal piglet jejunal IPEC-J2 cell monolayers were treated with arginine, reversible and irreversible nitric oxide synthase inhibitors, and were exposed to hypoxia.  
- Intestinal tight junctions were assessed by serial measurements of transepithelial electrical resistance, flux of inulin-fluorescein isothiocyanate, and immunofluorescent staining of tight junction proteins. | - Arginine and citrulline were protective against hypoxia related damage.  
- At the final time-point (14 hours) the mean transepithelial electrical resistance ratio (transepithelial electrical resistance compared to baseline) for arginine + hypoxia and citrulline + hypoxia was significantly higher than hypoxia alone  
- Both arginine and citrulline were associated with decreased inulin flux across hypoxic monolayers and qualitatively preserved tight junction proteins.  
- Irreversible inhibition of nitric oxide synthases blocked this protective effect. | Biochemistry  
Therapeutic significance of citrulline |
| 13  | Lee *et al.* (2012) | - Aim: To investigate the effects of long-term intra-duodenal supplementation of arginine on intestinal morphology, arginine-associated amino acid metabolism, and inflammatory responses in rats with intestinal ischaemia reperfusion.  
- Design: Male Wistar rats with or without three hours of ileal ischemia underwent duodenal cannulation for continuous infusion of formula with 2% arginine or commercial protein powder for 7 days  
- Serological examinations, plasma amino acid and cytokine profiles, and intestinal morphology were assessed | - Arginine supplementation decreased serum cholesterol and increased plasma arginine concentrations.  
- In rats with intestinal ischaemia reperfusion injury, arginine supplementation significantly decreased serum nitric oxide, plasma citrulline and ornithine, and the mucosal protein content of the ileum.  
- These results suggest that long-term intra-duodenal arginine administration may not have observable benefits on intestinal morphology or inflammatory response in rats with intestinal ischemia and reperfusion injury. | Biochemistry |
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| 14  | Marini (2012)    | • Aim: All possible enteral and plasma precursors of citrulline were studied in a mouse model during the postabsorptive and postprandial period using multitracer protocols  
• Design: Intragastric amino acids: 21 amino acids; Intravenous amino acids: proline, glutamine, arginine, glutamate  
• Three different models were used to interpret the stable isotope data. | • Dietary and plasma arginine were the main precursors for citrulline synthesis during feeding and plasma arginine during feed deprivation  
• Contribution of arginine was directly at the site of citrulline synthesis and through plasma ornithine, suggesting that ornithine amino transferase is a pivotal enzyme in this pathway | Biochemistry                    |
| 15  | Fijlstra et al.  (2011) | • Aim: to determine lactose digestion and absorption of its derivative glucose during mucositis  
• Design: Wistar rats were injected intravenously with methotrexate or 0.9% Sodium Chloride (controls)  
• Measurement of plasma citrulline level, harvestation of the small intestine to assess histology, myeloperoxidase level, glycohydrolase activity, immunohistochemical protein, mRNA expression | • During the experimental period, the absorption of lactose-derived glucose was 4.2-fold decreased in methotrexate-treated rats compared with controls  
• Lactose-derived glucose absorption correlated strongly with villus length ($\rho = 0.86$) and with plasma citrulline level ($\rho = 0.81$)  
• Plasma citrulline level correlated with the severity of mucositis as measured by villus length ($\rho = 0.90$) | Clinical Significance (Mucositis) |
| 16  | Marini et al.  (2011) | • Aim: To examine if arginase II plays a central role in the supply of ornithine for citrulline synthesis  
• Design: The contribution of dietary arginine, glutamine, and proline was determined by utilizing multitracer stable isotope protocols in arginase II knockout (AII−/−) and wild-type mice. | • The lack of arginase II resulted in a lower citrulline rate of appearance due to a reduced availability of ornithine; ornithine supplementation was able to restore the rate of citrulline production in arginase II knockout to levels comparable with wild-type mice  
• There were significant differences in the utilization of dietary citrulline precursors. The contribution of dietary arginine to the synthesis of citrulline was reduced from due to the lack of arginase II.  
• No enteral utilization of arginine was observed in arginase II knockout mice, and the contribution of dietary arginine through plasma ornithine was reduced in the transgenic mice  
• Dietary glutamine and proline utilization were greater in arginase II knockout than in wild-type mice  
• Most of the contribution of glutamine and proline was enteral rather than through plasma ornithine | Biochemistry                    |
| 17  | Puiman et al.  (2011) | • Aim: To examine if enteral arginine is a specific stimulus for neonatal intestinal blood flow and mucosal growth under conditions of total parenteral nutrition or partial enteral nutrition.  
• Design: Dose dependence and specificity of acute (3 hours) enteral arginine infusion on superior mesenteric artery blood flow in pigs fed total parenteral nutrition or partial enteral nutrition.  
• Investigation of whether chronic (4 days) arginine supplementation of partial enteral nutrition increases mucosal growth and if this was affected by treatment with the nitric oxide synthase inhibitor, N^o-nitro-arginine methyl ester.  
• A third group of pigs were fed partial enteral nutrition and were infused enterally with citrulline, glutamate, glutamine, or glucose as a control | • Plasma arginine and ornithine concentrations during the basal saline infusion were 80%–100% higher in partial enteral nutrition compared total parenteral nutrition pigs; this may have been due to either the arginine absorbed from the formula or endogenous synthesis by enterocytes.  
• Plasma concentrations of citrulline were higher, whereas those of glutamine, glutamate, and threonine were lower in partial enteral nutrition pigs than in total parenteral nutrition pigs across all arginine doses  
• Baseline superior mesenteric artery blood flow was 90% higher in the partial enteral nutrition pigs than in the total parenteral nutrition pigs, but was not affected by acute infusion individually of arginine, citrulline, or other major gut fuels  
• Supplementation arginine in partial enteral feeding modestly increases intestinal mucosal growth and was nitric oxide independent  
• Dietary arginine was a better precursor than citrulline for maintaining blood arginine | Biochemistry, Clinical Significance (total parenteral nutrition) |
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| 18  | Fang et al.      | **Aim:** To determine the effects of DL-2-hydroxy-4-methylthiobutyrate on the first-pass intestinal metabolism of dietary methionine and its extra-intestinal availability.  
**Design:** Barrows \( n = 6; \) aged 35 days; weight 8.6 kg, implanted with arterial, portal, mesenteric and gastric catheters, were fed a diet containing DL-methionine or of DL-2-hydroxy-4-methylthiobutyrate once hourly and infused intramesenterically with 1% \( \text{p-aminohippurate} \) and intragastrically with \( [1^{\text{13}}\text{C}]\text{methionine} \) at 7.0 \( \mu\text{mol/kg body weight per hour.} \)  
**Arterial and portal blood samples were taken at hourly intervals until 6 hours of tracer infusion and pigs was then killed for collection of muscle, intestine, liver and kidney samples. One-factorial arrangement** | Over the 6 hours period after the start of feeding, the average concentration of citrulline both in the arterial and portal plasma was higher \( (p < 0.05) \) in the of DL-2-hydroxy-4-methylthiobutyrate than in the DL-methionine group, and arterial plasma ornithine and taurine concentration was also higher \( (p < 0.05) \) in the DL-2-hydroxy-4-methylthiobutyrate than in the DL-methionine group | Biochemistry             |
| 19  | Geng et al.      | **Aim:** To determine developmental changes in mRNA and protein levels for N-acetylglutamate synthase (a key enzyme in synthesis of citrulline and arginine from glutamine/glutamate and proline) in the small intestine of suckling piglets.  
**Design:** The porcine N-acetylglutamate synthase gene was cloned using the real-time polymerase chain reaction method.  
**Results indicated that intestinal N-acetylglutamate synthase mRNA levels were lower in 7- to 28-day-old than in 1-day-old pigs.**  
**Immunohistochemical analysis revealed that N-acetylglutamate synthase protein was localized in enterocytes of the gut.**  
Notably, intestinal N-acetylglutamate synthase protein abundance declined progressively during the 28-day suckling period. |                                                                                                                                           | Developmental Biology    |
| 20  | Marini et al.    | **Aim:** To determine if other citrulline precursors can compensate when arginine is absent in the diet  
**Design:** Investigation of contributions of plasma and dietary precursors determined by using multitracer protocols in conscious mice infused i.e. either an arginine-sufficient diet or an arginine-free diet. | Plasma entry rate of citrulline and arginine did not differ between the 2 diet groups  
**Entry rate of ornithine was greater in the mice fed the arginine-sufficient diet than the arginine-free diet**  
**Greater utilization of plasma ornithine for the synthesis of citrulline in the mice fed the arginine-free diet than the arginine-sufficient diet**  
**Utilization of plasma arginine did not differ between the 2 diet groups for citrulline synthesis, either through plasma ornithine or at the site of citrulline synthesis**  
**The contribution of dietary proline to the synthesis of citrulline was mainly at the site of citrulline production, rather than through plasma ornithine**  
**Dietary glutamine was utilized only at the site of citrulline synthesis.**  
**Dietary glutamine and proline made a greater contribution to the synthesis of citrulline in mice fed the arginine-free diet but remained minor sources for citrulline production**  
**Plasma arginine and ornithine are able to support citrulline synthesis during arginine-free feeding** | Biochemistry             |
### Table 1.2  (Continued)

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| 21  | Siqueira et al.    | **Aim:** To determine serum citrulline concentrations in mice with hepatosplenic schistosomiasis, analyse the morphologic repercussions for the mucosa of the small intestine, correlate citrulline concentrations with morphometric changes in the intestinal mucosa, and evaluate the effect of splenectomy on citrulline concentration  | - The average citrulline concentration in the control group was greater than that in the experimental group both before and after splenectomy ($p < 0.001$).  
- In the experimental group, the villi had less height and area, and there was a smaller perimeter of the mucosal surface ($p = 0.003$, $p < 0.001$, and $p = 0.001$, respectively)  
- There was a direct correlation between citrulline concentration and the height and area of the villi ($p = 0.003$ and $p = 0.04$, respectively)  
- After splenectomy, there was a reduction in the mean citrulline concentration in the experimental group ($p = 0.009$). | **Clinical Significance**  
(Mucositis) |
|     | (2010)             | **Design:** 46 adult female albino Swiss mice were divided into two groups: Control (23 healthy mice) and experimental (23 mice with hepatosplenic schistosomiasis).  
**Blood samples** were collected for the analysis of plasma citrulline before and after splenectomy.  
**A segment of the jejunum** was resected for morphometric analysis. | **Results show that systems involved in citrulline transport are partly different from those involved in arginine transport.**  
**Kinetic analysis indicated the involvement of Na$^+$-dependent and Na$^+$-independent saturable transport components.**  
**Results show that systems involved in citrulline transport are partly different from those involved in arginine transport.** | **Biochemistry** |
| 22  | Berkeveld et al.   | **Aim:** To determine how, in piglets that have been subjected to intermittent suckling, age at weaning and the duration of the preceding intermittent suckling period contribute to postweaning adaptation through effects on feed intake, growth, and gut characteristics  
**Design:** All piglets had ad libitum access to creep feed from day 7. Litters were subjected to conventional weaning or to 1 of 3 intermittent suckling regimens. In conventional weaning, litters ($n = 29$) had continuous access to the sow until weaning (day 26, day 0 = farrowing). During intermittent suckling, litters had access to the sow between 16:00 and 06:00 hours. | - Weaning reduced plasma citrulline concentrations resulting in reduced concentrations on day 2 and 8 postweaning compared with the values observed at weaning (overall $p = 0.01$ and $p < 0.001$).  
- Postweaning plasma citrulline concentrations were not different between treatments.  
- At weaning, plasma citrulline concentrations were negatively correlated to crypt depth (overall $r = -0.36$, $p = 0.022$). At day 2 postweaning, plasma citrulline concentrations were negatively correlated to crypt depth ($r = -0.46$, $p = 0.003$) and small intestinal empty weight ($r = -0.62$, $p = 0.004$).  
- No correlation was observed between plasma citrulline concentration at day 2 postweaning and the relative growth check, average daily gain, or average daily feed intake during the first 2 days postweaning.  
- At day 8 postweaning, plasma citrulline was correlated to SI length ($r = 0.37$, $p = 0.017$). | **Clinical Significance**  
(Mucositis) |
|     | (2009)             | | **Biochemistry** |
| 23  | Fu et al.          | **Aim:** To investigate whether SOM230, a novel, metabolically stable analog with broad receptor affinity, reduces intestinal injury and lethality in mice exposed to total body irradiation.  
**Design:** Male CD2F1 mice were exposed to 7.15 Gy total body irradiation. Twice-daily administration of SOM230 (1, 4 or 10 mg/kg per day) or vehicle was started either 2 days before or 4 hours after total body irradiation and continued for either 14 or 21 days. Parameters of intestinal and hematopoietic radiation injury, bacterial translocation, and circulating cytokine levels were assessed. | - SOM230 prolonged survival and reduced lethality after exposure to total body irradiation  
- SOM230 preserved the intestinal mucosa surface area after irradiation and attenuated radiation-induced bacterial translocation.  
- Plasma citrulline levels did not increase significantly after SOM230 administration. | **Clinical Significance**  
(Mucositis) |
|     | (2009)             | | **Biochemistry** |
| 24  | Bahri et al.       | **Aim:** To characterize L-$[14C]$-citrulline uptake by Caco-2 cells.  
**Design:** Caco-2 cells were cultured in a bicameral insert system. Inhibition studies were conducted in the presence of neutral, cationic, acidic and non-metabolized amino acids. | Citrulline uptake was pH-independent whereas the uptake rate was reduced in the absence of Na$.  
Kinetic analysis indicated the involvement of Na$^+$-dependent and Na$^+$-independent saturable transport components.  
Results show that systems involved in citrulline transport are partly different from those involved in arginine transport. | **Biochemistry** |
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| 25  | Berkeveld et al. (2008) | • Aim: To investigate whether plasma citrulline or intestinal fatty acid-binding protein concentrations might be used as longitudinal markers for small intestinal function in piglets after weaning.  
  • Selected piglets per litter were either weaned conventionally or remained with the sow.  
  • Plasma citrulline and intestinal fatty acid-binding protein concentrations were measured longitudinally in weaned and unweaned piglets, and related to intestinal absorption values (i.e., plasma mannitol and 3-xylose concentrations in a sugar absorption test) | • The absorption of 3-xylose was not different between treatments ($p = 0.83$). Mannitol absorption, however, was less in the weaned conventionally piglets compared with the piglets on sow ($p = 0.003$), with the nadir on day 4 postweaning.  
  • Weaning also reduced plasma citrulline concentrations in the conventionally weaned treatment compared with the sow treatment ($p < 0.001$). On day 4 and 7 postweaning, plasma citrulline concentrations of conventionally weaned piglets were less ($p < 0.001$ and $p = 0.0013$) than preweaning values.  
  • In the conventionally weaned treatment, plasma citrulline concentrations correlated with plasma mannitol concentrations at day 4 postweaning ($r = 0.89$, $p = 0.008$ and overall ($r = 0.76$, $p = 0.001$) | Biochemistry (Absorption) |
| 26  | Bjornvad et al. (2008) | • Aim: To investigate whether the potential beneficial metabolic effects of total parenteral nutrition, prior to the start of enteral feeding, could help preterm neonates to resist feeding-induced mucosal dysfunction and inflammation and to investigate whether the previously reported protective effect of colostrum depends on provision of species-specific colostrum  
  • Design: Intestinal mass and necrotizing enterocolitis lesions were first recorded in preterm pigs fed enterally (porcine colostrum, bovine colostrum, or formula for 20-40 hours), with or without a preceding 2- to 3-day total parenteral nutrition period ($n = 435$) | • Mucosal mass increased during total parenteral nutrition and further after enteral feeding to reach an intestinal mass similar to that in enterally fed pigs without total parenteral nutrition  
  • Further studies in 3-day-old total parenteral nutrition pigs fed enterally showed that formula feeding decreased villus height and nutrient digestive capacity and increased luminal lactic acid and necrotizing enterocolitis lesions, compared with colostrum (bovine or porcine, $p < 0.05$).  
  • Formula feeding decreased plasma arginine, citrulline, ornithine, and tissue antioxidants, whereas tissue nitric oxide synthetase and gut permeability increased, relative to colostrum (all $p < 0.05$) | Clinical Significance (Necrotizing enterocolitis) |
| 27  | Kerem et al. (2008)    | • Aim: To evaluate the effects of chlorella crude extract on intestinal adaptation in rats subjected to short bowel syndrome.  
  • Design: Wistar rats weighing 230-260 g with 75% small bowel resection  
  • Randomized and divided into Control group ($n = 10$): where 5% dextrose was given through a gastrostomy tube; Enteral nutrition group ($n = 10$): Isocaloric and isonitrogen enteral nutrition, study group ($n = 10$): chlorella species crude extracts was administrated through a gastrostomy tube  
  • Histopathologic evaluation, intestinal macosal protein and DNA levels, intestinal proliferation and apoptosis were determined in intestinal tissues, and total protein, albumin and citrulline levels in blood were studied | • In rats receiving chlorella crude extract, villus lengthening, crypt depth, mucosal DNA and protein levels, intestinal proliferation, and serum citrulline, protein and albumin levels were found to be significantly higher than those in control group | Clinical Significance (Intestinal Resection) |
| 28  | Mittal et al. (2008)   | • Aim: To evaluate the effects of three nonsteroidal anti-inflammatory drugs with varying cyclooxygenase selectivities on the small intestinal antioxidant enzyme status and surface characteristics during 1,2-dimethylhydrazine administration.  
  • Design: Male Sprague-Dawley rats were divided into five different groups: Group 1 (control, vehicle treated); group 2 (1,2-dimethylhydrazine treated); group 3 (1,2-dimethylhydrazine + aspirin); group 4 (1,2-dimethylhydrazine + celecoxib); group 5 (1,2-dimethylhydrazine + etoricoxib) | • In comparison to 1,2-dimethylhydrazine-treated group, both celecoxib and etoricoxib showed significant changes.  
  • The celecoxib-treated group registered a significant increase ($p < 0.01$) in the citrulline level, whereas the etoricoxib showed highly significant ($p < 0.001$) increase. The aspirin-treated group showed a nonsignificant rise in the citrulline level | Clinical Significance (Cancer) |
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| 29  | Vissers et al. (2008) | • Aim: To investigate how the interorgan pathway for de novo arginine production is affected by the presence of malignant tumour and/or surgical trauma  
• Design: Controls and methylcholanthrene-sarcoma-bearing mice were studied, both with and without undergoing laparotomy (n = 9-13 per group). One day after laparotomy, amino acid fluxes across the hindquarter, intestine, liver, and kidney were studied (Male FVB mice).  
• Findings: In methylcholanthrene-sarcoma-bearing mice, decreased de novo arginine production was accompanied by the presence of hepatic citrulline uptake, whereas tumour-bearing mice subjected to surgical trauma showed concomitant decreased intestinal citrulline output. | Clinical Significance (Cancer) |
| 30  | Nadalin et al. (2007) | • Aim: To investigate the role of plasma citrulline levels as marker of acute cellular rejection after small bowel transplantation.  
• Design: Eighteen German landrace pigs were used and divided into three groups. Group 1, autologous small bowel transplantation (n = 4) as control; group 2, allogeneic small bowel transplantation without immunosuppression (n = 7) and group 3, allogeneic small bowel transplantation with immunosuppression (n = 7).  
• Findings: Acute cellular rejection was differentiated into indeterminate, mild, moderate and severe  
• An acute cellular rejection onset occurred generally from postoperative day 4 both in group 2 and group 3 as mild form and developed differently in the two groups: moderate to severe in group 2 and indeterminate to mild in group 3.  
• A significant decline of plasma citrulline occurred only in cases of moderate and severe acute cellular rejection, but not in cases of indeterminate and mild acute cellular rejection.  
• Plasma citrulline level failed as a marker in the early diagnosis of acute cellular rejection and became reliable only when advanced mucosal damage was present. | Clinical Significance (Transplantation) |
| 31  | Tsuchioka et al. (2006) | • Aim: To ascertain whether simultaneous administration of glutamic acid and taurine to patients on total parenteral nutrition could improve intestinal mucosal atrophy and suppress bacterial translocation.  
• Design: A 5-day total parenteral nutrition study was conducted in 5-week-old Sprague-Dawley rats.  
• Findings: Commercially available glutamic acid was used for total parenteral nutrition in group G and was enhanced with taurine in group GT. Oral nutrition was provided in controls  
• Mucosal thickness and villus height in the small intestine were lower for group with glutamic acid than for controls and glutamic acid/taurine  
• Arginine and citrulline levels in the groups with glutamic acid/ glutamic acid/taurine were lower than in controls  
• Citrulline concentration was lower in group with glutamic acid than in groups with glutamic acid/taurine and controls | Biochemistry, Clinical Significance |
| 32  | Urschel et al. (2006) | • Aim: To determine the most effective arginine precursor in 1-week-old enterally fed piglets.  
• Design: Piglets were administered either an arginine-deficient (basal) diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline or arginine for 5 days  
• Daily blood samples were taken and indicators of whole-body arginine status including plasma amino acid, ammonia, and urea concentrations were measured.  
• Findings: Piglets fed the basal diets supplemented with citrulline and arginine had lower plasma ammonia and urea concentrations (p < 0.05) and higher plasma arginine concentrations (p < 0.0001) and arginine fluxes (p < 0.05) than piglets fed the other 3 diets.  
• Piglets fed the basal diets supplemented with citrulline and arginine had a lower proline to arginine conversion (p < 0.05).  
• During first-pass splanchnic metabolism, 52% of the dietary arginine was extracted, and this extraction was not affected by whole-body arginine status (p > 0.05).  
• These data indicate that citrulline, but not ornithine or proline, is an effective arginine precursor, and that either citrulline formation or availability appears to limit arginine synthesis in neonatal piglets. | Biochemistry |
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<td>33</td>
<td>Boelen <em>et al.</em> (2005)</td>
<td>To quantify the interorgan relationship of exogenous L-glutamine or glutamine dipeptide, by enteral or parenteral route, contributing to intestinal citrulline and renal de novo arginine synthesis in mice.</td>
<td>Quantitatively, more de novo L-[2, 15N]citrulline was produced when free L-[2, 15N]glutamine was given than when L-alanine-L-[2, 15N]glutamine, whereas renal de novo L-[2, 15N]arginine was similar in all groups.</td>
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<td>34</td>
<td>Lardy <em>et al.</em> (2004)</td>
<td>To understand the metabolic changes underlying early adaptation after massive intestinal resection.</td>
<td>Both the basal rate of citrulline synthesis and the rate of citrulline synthesis that was stimulated by were similar.</td>
<td>Biochemistry, Clinical Significance (Intestinal Resection)</td>
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<td>35</td>
<td>Osowska <em>et al.</em> (2004)</td>
<td>To test the hypothesis that citrulline, which is not taken up by the liver and is the major precursor of arginine, should be a good candidate to generate arginine and improve nutritional status in massive intestinal resection.</td>
<td>Arginine concentration was higher (p &lt; 0.05) in plasma and muscle in the citrulline group than in the three other groups. Plasma levels of arginine were 110 (±12), 79 (±22), and 228 (±13) μmol/L in the sham, control, arginine, and citrulline groups respectively. Arginine concentrations in the gastrocnemius were: 0.15 (±0.02), 0.16 (±0.02), 0.40 (±0.05), and 0.94 (±0.20) μmol/g, respectively. Citrulline preserved nitrogen balance in resected rats but not in arginine supplemented rats.</td>
<td>Biochemistry, Clinical Significance (Intestinal Resection)</td>
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<td>36</td>
<td>Dekaney <em>et al.</em> (2003)</td>
<td>To measure enzymatic activities of carbamoyl phosphate synthase I, ornithine carbamoyltransferase, and pyrroline-5-carboxylate reductase, in ex vivo preparations, to determine whether functional enzymes exist within the porcine foetal small intestine, throughout gestation, to synthesize both citrulline and proline. To determine cell compartment-specific expression of the enzymes within the small intestine.</td>
<td>The activities of all three enzymes increased from day 30 to day 110 of gestation, and in situ hybridization demonstrates that 1) carbamoyl phosphate synthase I and ornithine carbamoyltransferase genes are expressed in distinct patterns and are confined to the mucosal epithelium and 2) pyrroline-5-carboxylate reductase mRNA is present in mucosal epithelium and lamina propria of the foetal porcine small intestine from day 30 to day 110 of gestation. The presence of carbamoyl phosphate synthase I and ornithine carbamoyltransferase in conjunction with the presence of ornithine aminotransferase suggests that the foetal porcine small intestine is capable of synthesizing citrulline from pyrroline-5-carboxylate.</td>
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| 37  | Lutgens et al.   | • Aim: To investigate whether citrulline can be used for quantifying radiation-induced epithelial cell loss  
• Design: NMRI mice were subjected to single-dose whole body irradiation. The time course of citrullinemia was assessed up to 11 days after irradiation. A dose-response relationship was determined at 84 hours after irradiation.  
• Citrullinemia was correlated with morphologic parameters at this time point and used to calculate the dose-modifying factor of glutamine and amifostine on acute small bowel radiation damage | • After whole body irradiation, a time- and dose-dependent decrease in plasma citrulline level was observed with a significant dose-response relationship at 84 hours.  
• At this time point, citrullinemia significantly correlated with jejunal crypt regeneration ($p < 0.001$) and epithelial surface lining ($p = 0.001$).  
• A dose-modifying factor of 1.0 and 1.5 was computed at the effective dose 50 level for glutamine and amifostine, respectively.  
• A linear correlation (Pearson’s $r = 0.77$) between plasma citrulline levels and a representative parameter for epithelial cell mass, i.e., the epithelial surface lining | Clinical Significance  
(Mucositis) |
| 38  | Bloomfield et    | • Aim: To investigate, in the late-gestation ovine fetus: 1) amino acid concentrations in blood and amniotic fluid, 2) the effects of intrauterine growth restriction induced by placental embolization on these concentrations, 3) foetal gut uptake of glutamine in healthy and intrauterine growth restriction foetuses, and 4) the effects of intraamniotic insulin-like growth factor-I treatment on these parameters.  
• Design: Foetuses (sheep) were randomly assigned to control ($n = 9$), Intrauterine Growth Restriction + saline ($n = 9$), or Intrauterine Growth Restriction + insulin-like growth factor-I ($n = 11$) groups. Intrauterine Growth Restriction was induced by uteroplacental embolization from 114 to 119 days (term = 145 days). Intrauterine Growth Restriction foetuses received daily intraamniotic injections of saline or insulin-like growth factor-I (20 µg/day) from 120 to 130 days. | • The authors described amino acid concentrations in the blood and amniotic fluid of the late-gestation foetal sheep and the changes that occur with Intrauterine Growth Restriction induced by uteroplacental embolization.  
• They demonstrated for the first time that the foetal gut also uses glutamine as a fuel and releases citrulline, possibly arising from the conversion of glutamine. | Biochemistry |
| 39  | Dekaney et al.   | • Aim: To determine ornithine aminotransferase enzymatic activity and mRNA expression in the intestine of foetal pigs from 30 to 110 days of gestation  
• Design: Foetuses were collected from crossbred (Duroc × Hampshire × Yorkshire) gilts at defined stages of gestation.  
• Ornithine aminotransferase enzyme activity was determined in homogenates of foetal small intestines from days 30, 35, 45, 60, 90, and 110 of gestation | • Enzymatic activity peaked at day 45 of gestation and increased again between day 60 and 110 of gestation.  
• At 30 and 35 days of gestation, ornithine aminotransferase mRNA expression was detected throughout the mucosal epithelium of the small intestine. Throughout the remainder of gestation, ornithine aminotransferase expression was notably higher in the villus epithelium than in the crypt epithelium.  
• The presence of ornithine aminotransferase in the small intestinal epithelium throughout gestation suggests that the porcine small intestine is capable of interconverting ornithine and pyrroline-5-carboxylate during foetal development | Biochemistry,  
Developmental Biology |
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| 40  | Wu *et al.* (2000) | • Aim: To determine whether a cortisol surge mediates the enhanced expression of intestinal citrulline-synthetic enzymes during weaning.  
• Design: Jejunal enterocytes were prepared from 29-days-old weanling pigs treated with or without metyrapone (an inhibitor of cortisol synthesis), or from age-matched unweaned pigs.  
• The mRNA levels and activities of phosphate-dependent glutaminase, pyrroline-5-carboxylate synthase, ornithine aminotransferase, carbamoyl-phosphate synthase I and ornithine carbamoyltransferase were determined | • The mRNA levels for phosphate-dependent glutaminase, pyrroline-5-carboxylate synthase, ornithine aminotransferase and ornithine carbamoyltransferase were 139%, 157%, 102% and 55% higher, respectively, in weanling pigs compared with suckling pigs.  
• The activities of phosphate-dependent glutaminase and pyrroline-5-carboxylate synthase were 38% and 692% higher, respectively, in weanling pigs compared with unweaned pigs, but the activities of ornithine aminotransferase, carbamoyl-phosphate synthase I and ornithine carbamoyltransferase did not differ between these two groups of pigs.  
• The effects of metyrapone administration to weanling pigs were as follows: 1) prevention of a cortisol surge, 2) abolition of the increases in both mRNA levels and activity of pyrroline-5-carboxylate synthase, 3) no alteration in the mRNA levels and activities of phosphate-dependent glutaminase and carbamoyl-phosphate synthase I, 4) increases in the mRNA levels for ornithine aminotransferase (216%) and ornithine carbamoyltransferase (39%) and in ornithine aminotransferase activity (30%), and 5) prevention of the increase in intestinal synthesis of citrulline from glutamine. | Biochemistry, Developmental Biology |
| 41  | Dillon *et al.* (1999) | • Aim: To determine whether lactate is an inhibitor of intestinal synthesis of citrulline and arginine from proline  
• Design: Jejunum was obtained from 14-day-old suckling pigs for preparation of enterocyte mitochondria and metabolic studies  
• Mitochondria were used for measuring proline oxidase activity in the presence of 0-10 mM L-lactate  
• Enterocytes were incubated at 37°C for 30 minutes in Krebs bicarbonate buffer (pH 7.4) containing 5 mM D-glucose, 2 mM L-glutamine, 2 mM L-[U-14C]proline, and 0, 1, 5, or 10 mM L-lactate | • Kinetics analysis revealed non-competitive inhibition of intestinal proline oxidase by lactate (decreased maximal velocity and unaltered Michaelis constant).  
• Lactate had no effect on either activities of other enzymes for arginine synthesis from proline or proline uptake by enterocytes but decreased the synthesis of ornithine, citrulline, and arginine from proline in a concentration-dependent manner | Biochemistry |
| 42  | Plauth *et al.* (1999a); Plauth *et al.* (1999b) | • Aim: To examine whether the route of glutamine administration and the simultaneous availability of glucose affect intestinal glutamine metabolism.  
• Design: Net substrate exchange rates of glutamine and its nitrogenous products were measured in the isolated vascularly and luminally perfused rat small intestine (a) as a function of glutamine provision from either the vascular or the luminal or simultaneously from both sides and (b) as a function of simultaneous availability of glucose from various routes | • When glutamine was provided from the lumen, only 19%-32% of absorbed glutamine appeared intact in the venous effluent  
• Glutamine consumption and the production of citrulline or ammonia decreased when glucose (vascular or luminal perfusate) became available in addition to glutamine.  
• The authors concluded that glutamine is utilized by the small intestine very efficiently regardless of the route of administration being enteral or parenteral. The two routes can be used interchangeably to provide the intestinal mucosa with glutamine. Glucose and glutamine may partially substitute each other, most likely for the purpose as a metabolic fuel. | Biochemistry |
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| 43  | Davis and Wu (1998)      | • Aim: To determine the compartmentation and kinetics of urea cycle enzymes in porcine enterocytes  
  • Design: Pigs were offspring of Yorkshire × Landrace sows and Duroc × Hampshire boars  
  • At 60 days of age, the jejunum and liver were dissected from anesthetized pigs  
  • Enterocytes were incubated at 37°C for 30 minutes in the presence of 5 mM glucose, 0.2 mM NH₄Cl and plasma concentrations of all amino acids  
  • Except for ornithine carbamoyltransferase, Vₘₐₓ values of urea cycle enzymes were much lower in enterocytes than in the liver of pigs, and vice versa for their Kₘ values  
  • Carbamoyl phosphate synthase I and ornithine carbamoyltransferase were located exclusively in mitochondria, whereas argininosuccinate synthase and argininosuccinate lyase were found in the cytosol  
  • Arginase isozymes were present in both the cytosol and mitochondria of enterocytes, and differed in their sensitivity to heat inactivation  
  • Because of a low rate of ureagenesis in enterocytes compared with the liver, intestinal urea cycle enzymes may function primarily to synthesize citrulline  
  • The co-localization of Carbamoyl phosphate synthase I and ornithine carbamoyltransferase and a high activity of ornithine carbamoyltransferase in enterocyte mitochondria favours the intestinal synthesis of citrulline from ammonia, HCO₃⁻ and ornithine  
  • Low activities of cytosolic argininosuccinate synthase and argininosuccinate minimize the conversion of citrulline into arginine and therefore, the recycling of citrulline into ornithine via arginase in postweaning-pig enterocytes. These kinetic properties of intestinal urea cycle enzymes maximize the net synthesis of citrulline from glutamine and explain the release of large amounts of citrulline by the pig small intestine. | Biochemistry |
| 44  | de Jonge et al. (1998)    | • Aim: To study the expression of ornithine cycle enzymes in the rat small intestine during perinatal development  
  • Design: The spatiotemporal patterns of expression of ornithine aminotransferase, carbamoylphosphate synthetase, ornithine transcarbamoylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase mRNAs were studied by Northern blot analysis and in situ hybridization  
  • The expression of carbamoylphosphate synthetase and argininosuccinate synthetase protein was studied by immunohistochemistry  
  • Before birth, the developmentally more mature proximal loops of the intestine expressed the mRNAs at higher concentrations than the more distal loops  
  • After birth, mRNAs of argininosuccinate synthetase and argininosuccinate lyase, the enzymes that metabolize citrulline to arginine, were detectable only in the upper part of the villi, whereas the other mRNAs were concentrated in the crypts  
  • The distribution of argininosuccinate synthetase protein corresponded with that of the mRNA, whereas carbamoylphosphate synthetase protein was present in all enterocytes of the crypts and villi. Hepatic arginase mRNA could not be detected in the enterocytes. The spatial distribution of the respective mRNAs and proteins along the villus axis of the suckling small intestine indicates that the basal enterocytes synthesize citrulline, whereas the enterocytes in the upper half of the villus synthesize arginine. | Biochemistry, Developmental Biology |
| 45  | Selamnia et al. (1998)    | • Aim: To determine whether L-citrulline present in the culture medium a precursor of L-arginine in HT-29 cells, newly synthesized L-arginine is used for the production of urea and L-ornithine  
  • Design: human adenocarcinoma cell line HT-29  
  • Cell incubation in the presence of radioactive precursors  
  • This study demonstrates that the polyamine precursor L-ornithine in HT-29 cells can (in addition to extracellular source and intracellular synthesis from L-arginine) be provided by the stepwise conversion of L-citrulline  
  • L-arginine derived from L-citrulline is not produced, even in the presence of D-glucose which limits the flux of L-arginine in the arginase pathway, in sufficient quantities to fulfill arginine needs for protein synthesis in an arginine-free culture medium. | Biochemistry |
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| 46  | Flynn and Wu (1997) | • Aim: To determine whether cortisol plays a role in arginine and glutamine metabolism in enterocytes and, more specifically, whether cortisol regulates metabolic changes in these cells during weaning.  
• Design: Twenty-eight 21-day-old suckling pigs were randomly assigned to one of four groups (7 animals in each) and received intramuscular injections of vehicle solution (sesame oil) (control group), hydrocortisone 21-acetate (25 mg/kg body weight), RU-486 (10 mg/kg body weight) (a potent blocker of glucocorticoid receptors), or hydrocortisone 21-acetate plus RU-486. | • Compared with control, cortisol administration increased:  
1) the activities of argininosuccinate lyase and arginase and the production of CO₂, ornithine, and proline from arginine  
2) pyrroline-5-carboxylate synthase activity and the formation of glutamate, alanine, aspartate, ornithine, citrulline, proline, and CO₂ from glutamine in enterocytes. | Biochemistry, Developmental Biology |
| 47  | Guihot et al. (1997) | • Aim: to assess the effects of an elemental diet compared with a complex diet on L-arginine metabolism in rat isolated enterocytes and its modulation by L-glutamine.  
• Design: Rats were fed the elemental diet or the control diet for 14 days.  
• Villus enterocytes then were isolated, and metabolic capacities or enzyme activities were assessed | • The incubation of enterocytes isolated from controls with 0.1 mmol/L L-[U-¹³C]-arginine led to the production of 125 ± 25 pmol L-citrulline/10⁶ cells per 30 minutes. This production showed a twofold increase in the presence of 2 mmol/L L-glutamine.  
• In the lemental diet group, L-citrulline synthesis from L-arginine was markedly lower in the absence or in the presence of L-glutamine. This coincided with lower carbamoylphosphate synthase I activity and carbamoylphosphate content of enterocytes. Other L-arginine and L-glutamine metabolic pathways were not affected.  
• Similar results were obtained when the elemental diet was administered continuously through a gastric catheter or fed by mouth. | Biochemistry, Developmental Biology |
| 48  | Wu (1997) | • Aim: To test that proline is an important substrate for the synthesis of citrulline and arginine in pig enterocytes.  
• Design: The synthesis of ornithine, citrulline, and arginine from proline was quantified in enterocytes of newborn (0-day-old) pigs, 2- to 21-day-old suckling pigs, and 29- to 58-day-old pigs weaned at 21 days of age.  
• Mitochondria were prepared from enterocytes for measurement of proline oxidase activity. | • Proline oxidase activity and rates of synthesis of citrulline and arginine from proline were high in enterocytes of new-born pigs and markedly decreased in 7-day-old pigs.  
• With increasing piglet age from 7 to 21 days, enterocyte proline oxidase activity and rate of conversion of proline into citrulline progressively increased, but the values in 21-day-old pigs remained much lower than in new-born pigs.  
• The synthesis of ornithine, citrulline, and arginine from 0.5-5 mM proline was concentration dependent and required the addition of glutamine. About 80%-90% of utilized proline carbons were recovered in ornithine plus citrulline plus arginine, with CO₂ being a minor product. | Biochemistry |
| 49  | Wu et al. (1997) | • Aim: To determine whether endogenous synthesis of arginine plays a role in regulating arginine homeostasis in postweaning pigs.  
• Design: Pigs were fed a sorghum-based diet containing 0.98% arginine and were used for studies at 75 days of age (28.4 kg body weight).  
• Mitochondria were prepared from the jejunum and other major tissues for measuring the activities of Δ¹-pyrroline-5-carboxylate synthase and proline oxidase and of ornithine aminotransferase. | • The activities of pyrroline-5-carboxylate synthase, proline oxidase and ornithine aminotransferase were greatest in enterocytes among all of the tissues studied.  
• Incubation of enterocytes with gabaculine resulted in decreases (p < 0.05) in the synthesis of ornithine and citrulline from glutamine and proline.  
• When gabaculine was orally administered to pigs to inhibit intestinal synthesis of citrulline from glutamine and proline, plasma concentrations of citrulline and arginine decreased whereas those of alanine, ornithine, proline, taurine and branched-chain amino acids increased.  
• Results suggest an important role for endogenous synthesis of arginine in regulating arginine homeostasis in postweaning growing pigs. | Biochemistry |
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<td>50</td>
<td>Chen et al.</td>
<td>Aim: To examine the changes in intestinal and circulating amino acid metabolism after massive small bowel resection in rats treated with standard or glutamine-supplemented total parenteral nutrition. Design: Intestinal amino acid flux, circulating plasma aminogram, mucosal glutaminase activity and protein, and DNA content were measured 7 days after massive small bowel resection in rats receiving either standard or glutamine-supplemented total parenteral nutrition. Sham-operated rats and rats fed chow after enterectomy served as controls.</td>
<td>The uptake of glutamine and the release of citrulline by the remaining intestine was significantly decreased, with reduced mucosal glutaminase activity after small bowel resection in the Chow and standard-total parenteral nutrition groups. Glutamine supplementation resulted in significantly increased gut glutamine uptake compared with standard-total parenteral nutrition group ($p &lt; 0.01$). Mucosal glutaminase activity, mucosal protein, and DNA content was also increased by glutamine; however, the gut release of citrulline remained unchanged ($p &gt; 0.05$). The subsequent decrease in circulating arginine in the glutamine-supplemented total parenteral nutrition group compared with the standard-total parenteral nutrition group ($p &lt; 0.05$) was attributed to an insufficient exogenous supply. These findings show that glutamine-supplemented total parenteral nutrition improves mucosal growth and gut glutamine uptake after small bowel resection. The intestinal production of citrulline, which remained low in both total parenteral nutrition groups, may lead to an insufficiency of endogenous arginine synthesis. Both glutamine and arginine may be essential amino acids after small bowel resection.</td>
<td>Biochemistry, Clinical Significance (Intestinal Resection)</td>
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<td>51</td>
<td>Flynn and Wu</td>
<td>Aim: To test the hypothesis that endogenous arginine synthesis plays an important role in maintaining arginine homeostasis in neonatal pigs. Design: Gabaculine was used as a suicide inhibitor of ornithine aminotransferase Four-day-old suckling pigs received oral administration of 0.0 or 0.83 mg gabaculine/kg body weight every 4 hours during a 12-hour period from 6 A.M. to 6 P.M.</td>
<td>Gabaculine treatment decreased plasma concentrations of ornithine, citrulline, and arginine by 59%, 52%, and 76%, respectively, and increased those of glutamine and proline by 74% and 229%, respectively. Because pyrroline-5-carboxylate synthase was almost exclusively located in enterocytes of 4-day-old pigs, data suggest that the intestinal production of citrulline plays an important role in endogenous synthesis of arginine.</td>
<td>Biochemistry</td>
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<td>52</td>
<td>Wu</td>
<td>Aim: To determine a role of pentose cycle in the provision of nicotinamide adenine dinucleotide phosphate for the synthesis of citrulline and proline from glutamine in porcine enterocytes. Design: Enterocytes from 4-day-old pigs were incubated at 37°C for 0 to 30 minutes in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 2 mM glutamine and 5 mM glucose in the presence of 0, 0.1, or 0.25 mM dehydroepiandrosterone a potent inhibitor of glucose-6-phosphate dehydrogenase which is the key regulatory enzyme of pentose cycle.</td>
<td>Dehydroepiandrosterone inhibited the synthesis of ornithine, citrulline, arginine, and proline from glutamine in a concentration-dependent manner, but had no effect on the formation of citrulline and arginine from ornithine. However, dehydroepiandrosterone decreased the synthesis of proline from ornithine by 79%. Dehydroepiandrosterone had no effect on cellular adenosine triphosphate concentrations.</td>
<td>Biochemistry</td>
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<td>53</td>
<td>Wu et al.</td>
<td>Aim: To determine degradation via arginase, nitric oxide synthase, and arginine decarboxylase in enterocytes of the pig Design: Arginine degradation was quantified in enterocytes of 0-day-old (new-born) and 4- to 21-day-old suckling pigs and 29- to 58-day-old pigs weaned at 21 days of age.</td>
<td>Arginine degradation to CO$_2$, ornithine, or proline was negligible in enterocytes of new-born and suckling pigs and markedly increased in weaned pigs. In cells from new-born pigs, citrulline generation from arginine was greater than that of ornithine plus CO$_2$. Citrulline synthesis decreased during the first 2 weeks after birth and increased in weaned pigs to the value similar to that in newborn pigs. The synthesis of citrulline from arginine decreased by &gt; 88% in the presence of $N^\text{G}$-nitro-L-arginine. Nitric oxide synthase plays a quantitatively minor role in arginine degradation by enterocytes.</td>
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| 54  | Dugan et al. (1995)   | • Aim: To determine the roles of age and diet in the induction of citrulline synthesis from glutamine in enterocytes of weaned pigs.  
• Design: Enterocytes were prepared from the jejunum of suckling pigs (14-29 days old), 23- and 29-days-old pigs weaned at 21 days of age to either a conventional corn-soybean meal-based or a milk-based diet, and 23-days-old pigs weaned at 21 days of age and food-deprived for 2 days. | • The rate of citrulline production from glutamine was similar in enterocytes from 14-, 21- and 29-days-old suckling pigs, and was 10-fold greater in cells from 29-days-old weaned pigs.  
• After weaning, enterocytes from 23-days-old pigs fed the milk-based diet had a 33% higher rate of citrulline production from glutamine than cells from age-matched pigs fed a corn-soybean meal-based diet or food-deprived for 2 days.  
• Findings suggest that the major determinant of the induction of citrulline synthesis from glutamine in enterocytes of weaned pigs may not be age or change in diet, although the extent of the induction may be slightly influenced by diet composition. | Biochemistry                   |
| 55  | Wakabayashi et al. (1995) | • Aim: To investigate the effect of intestinal resection and arginine-free diet on rat physiology.  
• Design: Maintained rats with massively resected small intestine and those with transected intestines on either control or an arginine-free diet | • After 4 weeks, massively resected small intestine rats fed deficient diet lost weight by a mean of 46 g, whereas massively resected small intestine rats fed control diet and transected intestines rats fed control and deficient diet gained 30-96 g. Average nitrogen balance was 150, 60, 110, and -33 mg/day for transected intestines rats fed control and deficient diet, massively resected small intestine rats fed control and deficient diet, respectively.  
• The concentrations of arginine in skeletal muscle were 654, 163, 230, and 84 mmol/g, respectively, and those in plasma were 133, 50, 103, and 54 μM, respectively. The concentrations of citrulline in massively resected small intestine rats were halved compared with transected intestines rats irrespective of diet. | Biochemistry, Clinical Significance (Intestinal Resection) |
| 56  | Wu and Knabe (1995)   | • Aim: To quantify arginine synthesis in neonatal pig enterocytes and to elucidate the mechanism involved in the developmental change of intestinal arginine synthesis.  
• Design: Arginine synthesis was quantified in enterocytes from newborn (0-day-old) and 2- to 7-day-old suckling pigs. | • Arginine was found to be synthesized from glutamine in 0- to 7-day-old pig enterocytes, but the rates of arginine synthesis were three- to fourfold greater in 0- to 2-day-old pigs than in 7-day-old pigs.  
• The rates of metabolism of glutamine to citrulline were 2.5- to 3.5-fold greater in enterocytes from 0- to 2-day-old pigs than in cells from 7-day-old pigs, as were the rates of conversion of citrulline to arginine.  
• The activities of all enzymes that synthesize arginine from glutamine, except pyrroline-5-carboxylate synthase and argininosuccinate lyase, increased in enterocytes from 2-day-old pigs compared with 0-day-old pigs.  
• The activities of all these enzymes decreased by approximately 75% in 7-day-old pigs compared with 2-day-old pigs. Arginase activity was negligible in enterocytes from 0- to 7-day-old pigs, thus minimizing intestinal hydrolysis of newly synthesized arginine and maximizing the endogenous provision of arginine. | Biochemistry, Developmental Biology |
| 57  | Wu et al. (1995a)     | • Aim: To determine whether pyrroline-5-carboxylate synthase is deficient in chick enterocytes therefore resulting in the lack of synthesis of ornithine and citrulline from glutamine  
• Design: Post-weaning pig enterocytes, which are known to contain pyrroline-5-carboxylate synthase and to synthesize both ornithine and citrulline from glutamine, were used as positive controls. | • In chick enterocytes, glutamine was metabolized to NH₃, CO₂, glutamate, alanine and aspartate, but not to ornithine, citrulline, arginine or proline.  
• Likewise, there was no formation of citrulline, arginine, alanine or aspartate from ornithine in chick enterocytes.  
• No activity of pyrroline-5-carboxylate synthase or ornithine carbamoyltransferase was found in chick enterocytes, in contrast with cells from post-weaning pigs.  
• It was also demonstrated that the activity of ornithine aminotransferase in chick enterocytes was only 3% of that in cells from pigs. | Biochemistry, Developmental Biology |
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| 58  | Wu et al. (1995b)        | • Aim: To quantify glutamine utilization by developing pig enterocytes and to assess the relative importance of glutamine versus glucose as a fuel for these cells.  
• Design: Glutamine and glucose metabolism was studied in 0- to 21-day-old pig enterocytes. Cells were incubated at 37°C for 30 minutes in Krebs-Henseleit bicarbonate buffer (pH 7.4) in the presence of 2 mM [U-14C]glutamine with or without 5 mM glucose, or 5 mM [U-14C]glucose with or without 2 mM glutamine. | • Glutamine was metabolized to ammonia, glutamate, alanine, aspartate, CO2, citrulline, ornithine, and proline, whereas glucose was converted to lactate, pyruvate, and CO2 in pig enterocytes.  
• By day 14 after birth, the oxidation of glutamine and glucose as well as citrulline production had decreased by 90.5%-95%.  
• Arginine synthesis from glutamine occurred in cells from 0- to 7-day-old pigs but not 14- to 21-day-old ones | Biochemistry, Developmental Biology |
| 59  | Malo (1994)              | • Aim: To evaluate the fate of ornithine as well as the levels of substrates and products of metabolic pathways in normal and ornithine transcarbamylase-deficient mice (sparse-ur)  
• Design: Concentration of free amino acids was measured in small intestinal mucosa and serum during post-natal development. | • In control animals, ornithine, alanine and citrulline concentrations in intestinal mucosa increased as a function of age  
• Major changes were observed in sparse-ur mice: the ornithine level remained low in intestinal tissue, citrulline concentration was significantly decreased in both intestinal tissue and blood, circulating levels of arginine and essential amino acids were drastically reduced in sucklings while plasma glutamine increased after weaning | Biochemistry |
| 60  | Wakabayashi et al. (1994) | • Aim: To investigate whether the small intestine is or is not the major tissue for the synthesis of arginine and proline  
• Design: Comparison of effects of feeding arginine- and/or proline-deficient diets with those of a complete diet (Complete) in rats whose small intestine had been massively resected. | • Weight loss, negative nitrogen balance, and markedly reduced arginine concentration in the muscle observed in rats fed arginine deficient and arginine deficient but not proline deficient diet clearly indicate that arginine becomes a strictly essential amino acid in the rats with massive resection of the small intestine.  
• Plasma glutamine, citrulline in the muscle and plasma, urinary excretion of orotic acid and nitrate (to assess nitric oxide formation from arginine) were also decreased | Biochemistry, Clinical Significance (Intestinal Resection) |
| 61  | Wu et al. (1994a)        | • Design: Arteriovenous differences in the plasma concentrations of amino acids across the jejunum were studied in preweaning (14- to 21-days-old) and post-weaning (20- to 58-days-old) pigs in the postabsorptive state. | • Glutamine was the only amino acid that was extracted by the small intestine in both pre- and post-weaning pigs  
• The production of citrulline by the jejunum was low in preweaning pigs, but was threefold greater in the post-weaning pigs than in the preweaning pigs  
• Demonstration of uptake in vivo of glutamine and the release of arginine, alanine, citrulline, glutamate and proline by the small intestine of developing pigs | Biochemistry, Developmental Biology |
| 62  | Wu et al. (1994b)        | • Aim: to quantify citrulline synthesis from glutamine in enterocytes and to elucidate the pathways involved.  
• Design: The synthesis of citrulline from glutamine was quantified in enterocytes from pre-weaning (14-21 days old) and post-weaning (29-58 days old) pigs.  
• The cells were incubated at 37 °C for 30 minutes in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 0, 0.5, 2 and 5 mM glutamine. | • The rates of citrulline synthesis were low or negligible in enterocytes from 14-21-day-old pigs, but increased 10-20-fold in the cells from 29-58-day-old pigs  
• This marked elevation of citrulline synthesis coincided with an increase in the activity of pyrroline-5-carboxylate synthase with the animal’s post-weaning growth  
• Decreases in the activities of phosphate-dependent glutaminase, ornithine aminotransferase, ornithine carbamoyltransferase and carbamoyl-phosphate synthase were observed as the age of the pigs increased  
• The concentrations of carbamoyl phosphate in enterocytes from pre-weaning pigs were higher than, or similar to, those in the cells from post-weaning pigs. It is possible that the low rate of citrulline synthesis from glutamine in enterocytes from pre-weaning pigs was due to a limited availability of ornithine, rather than that of carbamoyl phosphate | Biochemistry, Developmental Biology |
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| 63  | Blachier et al. (1993) | • Aim: to answer whether de novo synthesis of L-arginine from L-glutamine, L-citrulline or L-ornithine is possible in enterocytes isolated from newborn pigs and whether newborn pig enterocytes are able to catabolize exogenous L-arginine into urea and L-ornithine.  
• Design: The capacity for L-arginine metabolism was studied in villus enterocytes isolated from pigs at birth, after 2-8 days suckling and after weaning. | • 40% of L-arginine synthesized de novo from L-citrulline were released into the incubation medium  
• In enterocytes isolated from post-weaned pigs, no significant production of L-arginine from either L-glutamine or L-ornithine was detected  
• In contrast, although the L-arginine production from L-citrulline was very low in post-weaned animals, it was significantly enhanced in the presence of L-glutamine, representing 23% of the production measured in suckling animals | Biochemistry,  
Developmental  
Biology |
| 64  | Vadgama and Evered (1992) | • Aim: To examine the characteristics of citrulline transport across the everted sacs of the rat small intestine.  
• Design: Male albino (Wistar) rats, 180-240 g body weight, were starved overnight but given water ad libitum. | • Optimal site of citrulline absorption is middle to lower small intestine  
• It shows active transport, and this transport is predominantly Na⁺ dependent.  
• Analog inhibition studies suggest that citrulline may share the neutral brush border system described for the mucosal brush border membranes of the rabbit jejunum or a system analogous to system ASC described for nonepithelial cells and for basolateral membranes of certain epithelia.  
• The rat small intestine has developed a specific carrier-mediated, Na⁺-dependent pathway for citrulline absorption. | Biochemistry |
| 65  | Blachier et al. (1991a) | • Aim: To examine the metabolic fate of L-arginine in isolated rat enterocytes, with emphasis on its conversion to L-ornithine, L-citrulline and polyamines  
• Design: Rat enterocytes were isolated from fed female albino rats and exposed to L-arginine | • L-Arginine was converted to L-citrulline either directly in a nicotinamide adenine dinucleotide phosphate-sensitive manner thought to be coupled with the generation of nitric oxide, or indirectly through the sequence of reactions catalysed by arginase and ornithine transcarbamylase.  
• A large fraction of L-citrulline and L-ornithine generated from exogenous L-arginine was released in the incubation medium.  
• The oxidative catabolism of L-arginine in enterocytes is quantitatively negligible relative to its conversion to L-citrulline and L-ornithine. | Biochemistry |
| 66  | Blachier et al. (1991b) | • Aim: To investigate more directly the presence of L-arginine:nicotinamide adenine dinucleotide phosphate-sensitve manner thought to be coupled with the generation of nitric oxide, or indirectly through the sequence of reactions catalysed by arginase and ornithine transcarbamylase.  
• Design: Enterocytes isolated from jejunum of large White pigs weighing 54 ± 4 kg (n = 8) were studies | • Since the production of labelled L-citrulline from L-arginine in pig enterocyte homogenates was markedly increased in the presence of nicotinamide adenine dinucleotide phosphate, it is proposed that  
• o The direct conversion of L-arginine to L-citrulline could be stimulated by the production of nicotinamide adenine dinucleotide phosphate from D-glucose in the pentose phosphate pathway. | Biochemistry |
| 67  | Edmonds et al. (1987) | • Aim: to determine the efficacy of ornithine or citrulline as precursors of arginine  
• Design: Pigs were individually fed an arginine-deficient, semipurified diet supplemented with 0.3% arginine or an equimolar quantity of ornithine or citrulline.  
• Pigs were again fed the arginine-deficient, semipurified diet supplemented with 0.3% arginine or four times an isomolar quantity of ornithine. | • Supplemental arginine or citrulline increased rate and efficiency of weight gain, but ornithine addition was without effect.  
• Free arginine in plasma 3 hours post-prandial was increased by addition of either arginine or citrulline to the basal diet.  
• Arginine or citrulline addition to the diet increased arginine concentration in muscle tissue, but muscle ornithine was unresponsive to any of the supplements fed.  
• Arginine addition to the diet increased weight gain and feed efficiency, while ornithine supplementation was without effect. Plasma ornithine was increased by excess ornithine, while plasma citrulline was unaffected by supplemental arginine or ornithine. | Biochemistry |
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<td>68</td>
<td>Hurwitz and Kretchmer (1986)</td>
<td>* Aim: To localize the source of endogenous arginine during the newborn period, to determine the developmental patterns of the urea biosynthetic enzymes in the kidney and intestine of the mouse, and to initiate a study concerned with the fate of the newly synthesized arginine. * Design: Mice of the C57B1/6 strain were maintained on Purine mouse laboratory chow ad libitum. Only timed pregnancies were used for the study of foetuses.</td>
<td>At birth, the arginine-synthesizing enzymes in the kidney of the C57Bl/6 mouse are minimally developed, whereas in the intestine activity of carbamoyl-phosphate synthase is elevated and arginosuccinate synthase and lyase, usually present only in trace quantities in the adult intestine, are markedly increased in the newborn. * The study indicates that, at a time when no other endogenous source of arginine for protein synthesis is available, the intestine of the newborn C57Bl mouse is capable of synthesizing arginine from either citrulline or NH$_3$ and CO$_2$.</td>
<td>Biochemistry, Developmental Biology</td>
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<td>69</td>
<td>Uchiyama et al. (1981)</td>
<td>* Aim: To investigate the activity of N-acetylglutamate synthase in rat small intestinal mucosa. * Design: Wistar or Sprague-Dawley male rats, weighing 250-300 g, were maintained on a commercial laboratory chow containing 24% crude protein for 1-3 weeks before use.</td>
<td>N-Acetylglutamate synthase, had a high substrate specificity for L-glutamate and acetyl coenzyme A. Results suggest that a function of the intestinal N-acetylglutamate is to activate carbamoyl-phosphate synthase (ammonia) and to allow citrulline synthesis in the tissue.</td>
<td>Biochemistry</td>
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<td>70</td>
<td>Windmueller and Spaeth (1981)</td>
<td>* Aim: To assess the physiologic importance of the intestinal citrulline contribution and to re-evaluate the importance of the liver * Design: Measurement of citrulline uptake and release by isolated, perfused livers and, through surgical means and arteriovenous difference measurements, by various organs of the rat in vivo. * Osborne-Mendel males (260-450 g) were fed NIH-07 open-formula stock diet ad libitum. Fasted rats had their food withheld overnight.</td>
<td>No significant extraintestinal source of circulating citrulline was found. Renal citrulline uptake was equivalent to approximately 83% the rate of intestinal release; kidneys, in turn, released arginine equivalent to approximately 75% of the citrulline taken up.</td>
<td>Biochemistry</td>
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<td>71</td>
<td>Herzfeld and Raper (1976)</td>
<td>* Aim: To measure in whole upper intestine, or in duodenum, small intestine and colon of adult rats the levels of 11 enzymes, most of them involved in the metabolism of ornithine * Design: The developmental formations in small intestine of arginase, ornithine aminotransferase, and ornithine transcarbamylase were compared with those in liver. * Changes with age (late gestation of adult) of the intestinal activities of pyrroline-5-carboxylate reductase, proline oxidase and glutamyl transpeptidase were also described.</td>
<td>The results suggest that the proximal part of the intestine is well endowed with enzymes involved in the conversion of ornithine to proline as well as to citrulline.</td>
<td>Biochemistry, Developmental Biology</td>
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<td>72</td>
<td>Raijman (1974)</td>
<td>* Aim: To investigate the subcellular distribution of ornithine carbamoyltransferase in the liver to establish whether its activity found in soluble fractions of rat and human liver reflects the distribution in vivo or artifacts occurring during the disruption of the tissue and the separation of the subcellular fractions. * Design: Male Wistar rats weighing approximately 125 g * For the measurement of ornithine carbamoyltransferase activity in other tissues, a radioactive assay utilizing $[^{14}C]$carbamoylphosphate was used.</td>
<td>Rat liver ornithine carbamoyltransferase appears to be located exclusively in the mitochondria. * Only the liver and the mucosa of small intestine contain significant amounts of ornithine carbamoyltransferase. * Activity in intestinal mucosa is less than one thousandth of that in liver.</td>
<td>Biochemistry</td>
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Table 1.3  Citrulline function in colonocytes.

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| 1   | Armstrong et al.      | - Aim: to assess aminoguanidine, a selective inhibitor of inducible nitric oxide synthase, with regard to its effectiveness as a nitric oxide inhibitor and as a modulator of inflammation in trinitrobenzene sulfonic acid-induced colitis.  
- Design: Colitis was induced in Wistar rats. Selective aminoguanidine and non-selective 1-nitro-arginine methyl ester inhibitors of nitric oxide synthase were given in the drinking water.  
- Colonic citrulline and arginine concentrations were assessed using high-performance liquid chromatography. The severity of colitis was assessed by a macroscopic scoring system. | - Both 1-nitro-arginine methyl ester and aminoguanidine successfully reduced nitric oxide synthesis. There was no evidence of substrate depletion in the colonic wall. Neither of the agents reduced the severity of colonic inflammation.  
- Oral administration of nitric oxide synthase inhibitors reduced nitric oxide synthesis in the colonic wall. |
| 2   | Chakder and Rattan    | - Aim: to further examine the role of L-citrulline recycling in the internal anal sphincter smooth muscle relaxation by the simultaneous determinations of tissue levels of L-arginine.  
- Design: Studies were performed on the internal anal sphincter smooth muscle strips obtained from opossums (Didelphis virginiana).  
- Isometric tension and L-arginine levels of the tissues were measured under basal conditions, in the presence of electrical field stimulation and after treatment with different concentrations of arginine. | - The basal levels of L-arginine were found to be significantly higher in the internal anal sphincter (tonic smooth muscle) than in the rectal (phasic smooth muscle) smooth muscle.  
- Arginase caused a concentration-dependent attenuation of the internal anal sphincter relaxation caused by electrical field stimulation. L-Citrulline and L-arginine were equipotent in reversing the attenuation.  
- Both arginine (60 minutes pre-treatment) and continuous electrical field stimulation (tissues collected at the time of maximal recovery of the basal internal anal sphincter tone after the initial relaxation) caused significant decreases in L-arginine levels.  
- The decreases in the levels of L-arginine were restored by the exogenous administration of either L-arginine or L-citrulline.  
- The restoration of L-arginine levels by L-citrulline but not by L-arginine was selectively blocked by L-glutamine. |
| 3   | Rattan and Chakder    | - Aim: To investigate the possibility of L-citrulline recycling in the maintenance of nitrergic neurotransmission in the opossum internal anal sphincter smooth muscle strips.  
- Design: Responses to nonadrenergic, noncholinergic nerve stimulation by electrical field stimulation on the basal internal anal sphincter tension were recorded before and after the nitric oxide synthase inhibitor Nω-nitro-L-arginine, Nω-nitro-L-arginine plus L-citrulline, or L-arginine.  
- During continuous electrical field stimulation, when the basal internal anal sphincter tone after the initial relaxation had recovered to almost pre-electrical field stimulation levels, the effects of L-citrulline or L-arginine were examined before and after L-glutamine, which is a putative blocker of L-citrulline uptake. | - Inhibition of nonadrenergic, noncholinergic nerve-mediated internal anal sphincter relaxation by Nω-nitro-L-arginine was reversed by L-citrulline as well as L-arginine.  
- L-Citrulline and L-arginine caused concentration-dependent relaxation of the internal anal sphincter tone recovered during the prolonged electrical field stimulation.  
- L-Glutamine blocked the responses of L-citrulline but not of L-arginine. Furthermore, L-glutamine increased the speed of recovery of internal anal sphincter tone during continuous electrical field stimulation.  
- L-citrulline recycling may be responsible for the maintenance of internal anal sphincter relaxation during frequent short-train and prolonged nonadrenergic, noncholinergic nerve stimulation. |
Table 1.3  (Continued)

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| 4   | Shuttleworth et al. (1995); Shuttleworth and Sanders (1996); Shuttleworth et al. (1997) | • Aim: Investigation of the contribution of nitric oxide to inhibitory neuromuscular transmission in murine proximal colon and the possibility that citrulline is recycled to arginine to maintain the supply of substrate for nitric oxide synthesis.  
• Design: Intracellular microelectrode recordings were made from circular smooth muscle cells in the presence of nifedipine and atropine (both 1 μM). | • L-Nitro-arginine-methyl ester (100 μM) selectively abolished the slow component of i. j. p. s.  
The effects of L-Nitro-arginine-methyl were reversed by L-arginine (0.2-2 mM) but not by D-arginine (2 mM).  
L-Citrulline (0.2 mM) also reversed the effects of L-Nitro-arginine-methyl, and this action was maintained during sustained exposures to L-citrulline (0.2 mM). This may reflect intraneuronal recycling of L-citrulline to L-arginine.  
Higher concentrations of L-citrulline (e.g. 2 mM) had time-dependent effects. Brief exposure (15 minutes) reversed the effects of L-Nitro-arginine-methyl, but during longer exposures (30 minutes) the effects of L-Nitro-arginine-methyl ester gradually returned. In the continued presence of L-citrulline, L-arginine (2 mM) readily restored nitrergic transmission, suggesting that during long exposures to high concentrations of L-citrulline, the ability to generate arginine from citrulline was reduced.  
• Recycling of L-citrulline to L-arginine may sustain substrate concentrations in support of nitric oxide synthesis and this pathway may be inhibited when concentrations of L-citrulline are elevated. |
| 5   | Berrino et al. (1995) | • Aim: Nitric oxide synthase activity in rat isolated longitudinal muscle myenteric plexus preparations of the small intestine was determined by measuring the accumulation of 3H-L-citrulline during 30 minutes incubation with 3H-L-arginine  
• Design: Sprague-Dawley rats were used. The whole small intestine was dissected and longitudinal muscle strips with the longitudinal muscle myenteric plexus attached were prepared | • Intermittent electrical field stimulation caused a threefold increase in 3H-L-citrulline accumulation.  
The nitric oxide synthase inhibitor N\(^\mathrm{ω}\)-nitro-L-arginine methyl ester reduced the spontaneous accumulation of 3H-L-citrulline by 65% and prevented the electrically evoked increase.  
During incubation with 3H-L-arginine tissue levels of 3H-L-citrulline in rat isolated longitudinal muscle myenteric plexus preparations, but not accumulation in incubation media may be used as a biochemical marker of the activity of nitrergic intestinal neurons which appear to be inhibited via muscarinic receptors. |
| 6   | Grider and Jin (1993) | • Aim: Vasoactive intestinal peptide release and L-\([\text{3H}]\)citrulline production were examined in ganglia isolated from the myenteric plexus of guinea-pig intestine.  
• Design: Ganglia were isolated from the ilium of the guinea-pig.  
Sheets of longitudinal muscle layer with adherent myenteric plexus were removed from segments of intestine (223 cm long) by tangential stroking. | • The nicotinic agonist 1,3-dimethyl-4-phenylpiperazinium stimulated vasoactive intestinal peptide release and L-\([\text{3H}]\)citrulline production; the latter was considered an index of nitric oxide production.  
Both vasoactive intestinal peptide release and L-\([\text{3H}]\)citrulline production were abolished by tetrodotoxin, hexamethonium, and the nitric oxide synthase inhibitor, N\(^\mathrm{ω}\)-nitro-L-arginine.  
The pattern of stimulation by nitric oxide and inhibition by N\(^\mathrm{ω}\)-nitro-L-arginine implied that vasoactive intestinal peptide release is facilitated by and may be dependent on nitric oxide production.  
• The study provides the first direct evidence of nitric oxide production from enteric ganglia. |
Chapter 1: Introduction

1.6 Figures

The Medical Age.
Vol. XII. DETROIT, FEBRUARY 26th, 1894. No. 4.

GLYCERIN AND CITRULLIN SUPPOSITORIES.

Suppositories, as a rule, are ineffective where constipation is due to febrile diseases, to affections of the brain and spinal cord, or to mechanical obstruction of the intestinal circulation. In such cases, however, the same suppositories, fortified by the addition of Citrullin, will secure in most satisfactory manner the desired result; the latter are

Figure 1.1. Passage from The Medical Age 1894;12:115, mentioning citrullin[e] suppositories.
Figure 1.2. Drawing of *C. colocynthis* (Culbreth, 1917, p. 585).
Figure 1.3. Citrulline structure. The characteristic group of citrulline, ureide group (NH$_2$-CO-NH), is shown in the box.
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Figure 1.4. The first page from the article by Koga and Ohtake (1914) in the *Journal of the Tokyo Chemical Society*. To be read from right to left and from up to down.
Figure 1.5. Overview of synthesis of L-citrulline and L-arginine from L-glutamine. Catabolism of L-arginine to L-ornithine/urea or L-citrulline/nitric oxide, production of polyamines, and anabolism and catabolism of proline are also shown. L-arginine enters the urea cycle and L-ornithine is reproduced. L-arginine also is converted to citrulline through the effect of nitric oxide synthases. Abbreviations: ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; Asp, aspartate; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OCT, ornithine carbamoyltransferase; P5CS, pyrroline-5-carboxylate synthase. Source: Romero et al. (2006); Cynober et al. (2010).
Figure 1.6. The fate of citrulline and the arginine/glutamine-citrulline-arginine cycle. Citrulline is produced at the enterocyte and is circulated to the kidney where it is broken down to arginine. Intestinal ornithine carbamoyltransferase is derepressed when protein intake is low. This process increases citrulline release and decreases arginine release in the portal vein, which increases arginine synthesis by the kidney and decreases liver ureagenesis, respectively. Abbreviations: Arg: arginine, Cit: citrulline, Gln: glutamine, Orn: Ornithine.
Figure 1.7. Flow chart of the systematic review of animal studies.
Chapter 2
Citrulline and Intestinal Function: Systematic Review and Meta-Analysis

2.1 Introduction

For this section, a systematic review of the literature was done. A comprehensive research in Pubmed/Medline, Scopus and Embase was conducted searching for articles on citrulline and the intestine in human populations. Certain conditions were identified in which citrulline has been used as a marker of intestinal function. However, it is not clear whether citrulline levels reflect intestinal function (notably absorption), enterocyte mass, both or other. The literature has been using this marker interchangeably but at the same time researchers are cautious about attributing to it an absolute interpretation.

Diagnostic markers are commonly used in the context of certain clinical conditions where variations in their levels indicate certain pathophysiological processes. However, certain markers have such a specificity that values outside normal range commonly indicate a limited number of clinical conditions, such that the differential diagnosis is narrow and hence clinical diagnosis of the condition is very likely. For example, in daily clinical practice, it is completely valid and reasonable to deduct that when C-reactive protein is over 5 mg/L – and quite strongly when over 10 mg/L – there is an inflammatory process in the human body (infection or other): it can also be likely deducted that when creatinine and urea surpass their upper normal limits (which differ between hospitals and populations), the patient has a possible acute kidney injury. Does citrulline currently maintain a place in clinical practice such that when it is out of a certain normal range [let it be (x, y) μmol/L], the patient has intestinal failure, insufficiency, malabsorption or dysfunction in general? I will leave this question unanswered for the time being and come back to it once the evidence is presented.

Hence, citrulline’s unique metabolism (production at the intestinal enterocyte) and complex findings from various studies compel me to submit the following questions (to myself but to the reader as well) – which I will try to answer at the end of the chapter and thesis. The questions are:
1. Is citrulline a successful indicator of enterocyte mass?
2. Is citrulline a successful marker of enterocyte absorption?
3. What clinical conditions has citrulline been utilized in as a marker?
4. Can a new conceptual theory be postulated regarding citrulline’s clinical utility?\(^1\)

I first describe the methods for this research literature synthesis and then move on to categorising the findings in sections for better understanding.

### 2.2 Methods

#### 2.2.1 Study Eligibility Criteria

The inclusion criteria for this systematic review were: any empirical study (abstract and full paper) describing investigation of citrulline in relation to the term intestinal function. Intestinal function was considered in a very broad term and could mean enterocyte mass, diarrhoea, absorption, or even deranged citrulline levels in correlation with a compromised gut. The only papers that were excluded were those whose object of investigation was not related to intestinal function. There was no restriction to language of papers and the types of interventions could include observational studies, randomized controlled trials, case series and case reports. For the meta-analysis papers had to provide sufficient data to produce an effect measure.

#### 2.2.2 Search Strategy and Terms

PRISMA guidelines and MOOSE checklist for systematic reviews and meta-analyses were used (Stroup et al., 2000; Moher et al., 2009). Electronic database searches were conducted in Google Scholar, Pubmed/Medline, Scopus, EMBASE and Cochrane Library with no year limits. Publisher databases were also searched (Sciencedirect.com, link.springer.com, Wiley Library Online, Highwire Press, Nature.com, Ovid, Cambridge University Press). The search keywords were: citrulline, intestine, gut, bowel, intestinal, mucositis, Crohn’s disease, short bowel, radiotherapy, cancer, sepsis, absorption, enterocyte, critically ill, and colitis. The date of search was up until 1 July 2015. The bibliographies from all included

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\(^1\) I give a preliminary note that I will introduce the term possible acute gut injury as a result of the findings.
manuscripts and hand searching of relevant gastroenterology and nutrition journals were used to identify further references.

2.2.3 Study Selection, Data Extraction and Quality Assessment

The resulting studies (in abstract form) were assessed against the inclusion criteria. When there was insufficient information available in the abstract, the full text was reviewed. Then, data were extracted from the selected studies including: author, year of publication, aim of the study, country, continent, sample size, mean age, male percentage, study design, results. The quality of studies (risk of bias) was assessed with elements from Cochrane Collaboration’s tool (Higgins et al., 2008) and the RTI Item Bank for Observational Studies (Viswanathan and Berkman, 2012; Viswanathan et al., 2013), which assess selection, attrition, detection and confounding biases. For the meta-analysis, studies were examined for p-values, means and standard deviations, correlation coefficients or other metrics depicting the association of citrulline with intestinal function. Metrics were converted to the standardized mean difference (SMD) (Lipsey and Wilson, 2001; Cooper et al., 2009) or mean difference (MD), where means and standard deviations for groups under comparison were identified (Borenstein et al., 2008); and/or correlation coefficients (CCs). Examples of outcomes included correlations of citrulline levels with small bowel length, with absorption tests/enteral caloric intake, differences in citrulline between tests groups and controls etc. Particularly, studies included satisfied the following criteria: a) for correlations of citrulline with small bowel length, only short bowel syndrome; b) for absorptive marker correlations, all patient groups c) for gastrointestinal disease severity, all patient groups. Where available, diagnostic accuracy data were also collected.

2.2.4 Statistical Analysis

Meta-analysis in medicine is any structured and systematic qualitative and/or quantitative integration of the results of several independent studies on a health problem (Jenicek, 1989). Three main reasons suggest performing a meta-analysis: statistical significance, clinical importance of the effect and consistency of the effects.

Quantitative analysis was performed with Stata 12.0 (StataCorp LP, Texas), Review Manager 5.3 (Cochrane Collaboration, Copenhagen) and SPSS 22.0 (IBM Corp.,
Armonk, NY). SMDs/MDs/CCs were extracted from studies when available. The strength of association was categorized as following: small, SMD = 0.2; moderate, SMD = 0.5; and large, SMD = 0.8; MD has units and reflects the units of the outcome under description; small, CC = 0.1; moderate, CC = 0.3; and large, CC = 0.5 (Cohen, 1988; Faraone, 2008). A random effects model was used to produce a pooled estimate of the SMDs/MDs/CCs. With the random effect model, studies are weighted by the inverse of their variance with $\tau^2$, taking into account the within study variance for estimating the correlation coefficient in each study and the between studies variance (e.g. because of different designs or methods used but also possible biological reasons) (DerSimonian and Laird, 1986). Statistical heterogeneity was assessed using Cochran’s $Q$ test, which examines the null hypothesis that all studies are evaluating the same effect (Higgins et al., 2003). Statistical significance for heterogeneity was set as $p \leq 0.10$. Heterogeneity was quantified using the $I^2$ statistic, indicating the percentage of total variation across studies that is due to heterogeneity rather than chance (Higgins et al., 2003). $I^2$ value of 0% was considered to indicate no observed heterogeneity whilst a value > 50% substantial heterogeneity (Higgins and Thompson, 2002; Huedo-Medina et al., 2006; Bowden et al., 2011; Fragkos et al., 2014). Heterogeneity was further investigated with subgroup analysis and meta-regression. Publication bias was assessed using funnel plots, Egger’s test, Begg’s test and Rosenthal’s number (Rosenthal, 1979; Begg and Mazumdar, 1994; Egger et al., 1997; Fragkos et al., 2016). A funnel plot was created for the clinical measures with more than 10 studies (Sterne et al., 2011). This is a scatter plot of the effect estimates from individual studies against a measurement of the study’s sample size or precision. Resemblance of a symmetrical inverted funnel supports that findings are due to sampling variation alone; thus, absence of bias (Sterne et al., 2011).

Regarding CCs, it is common practice not to perform syntheses on the correlation coefficient itself because the variance depends strongly on the correlation. Rather, the CC is converted to the Fisher’s $z$ scale (not to be confused with the $z$-statistics used with significance tests), and all analyses are performed using the transformed values. The results, such as the summary effect and its confidence interval, would then be converted back to correlations for presentation. The transformation from sample correlation $r$ to Fisher’s $z$ is given by $z = 0.5 \times \ln \left( \frac{1+r}{1-r} \right)$. The variance of $z$
is $V_i = \frac{1}{n-3}$ and the standard error is $SE_i = \sqrt{V_i}$, where $n$ is the sample size of the study. We then convert each of these values back to correlation units using $r = \frac{e^{2z_i} - 1}{e^{2z_i} + 1}$.

Regarding diagnostic accuracy data, following the robust construction of the diagnostic $2 \times 2$ tables, specificity, sensitivity, and 95% CI for each of the included studies was calculated. A hierarchical summary receiver operating curve (HSROC) model was fitted to provide a summary receiver operating curve and to allow derivation of pooled sensitivity and specificity estimates (Harbord et al., 2007). As suggested by the Cochrane Diagnostic Test Accuracy group (http://srdta.cochrane.org/), no analyses of study heterogeneity were performed, as these tests do not account for heterogeneity explained by phenomena, such as positivity threshold effects (Higgins et al., 2008).

### 2.3 Results

The next sections describe results per condition and/or function. Each section reports separately on condition and/or function with separate meta-analyses, where possible, including reports for quality assessment and other analyses where appropriate. The flow chart for studies is shown in Figure 2.1; from initially 463 studies, 131 were included in the systematic review and 63 in the meta-analyses performed. As a general description of papers, patients in the meta-analyses reached 4292 (mean 68, range 6-847) with mean age 31.6 years old, male percentage 50.9% and BMI 21.9 kg/m$^2$. Twenty three studies involved children and forty involved adults and the majority of studies were conducted in Europe (45 studies). Mean citrulline value from all studies was 23.2 $\mu$mol/L and citrulline was mostly measured with HPLC. Main findings from all studies are shown in Tables 2.1-2.6, grouped by condition.

Regarding overall quality assessment and risk of bias, there was a strong presence of detection bias and almost 50% confounding bias (Figures 2.2 and 2.3). Reporting bias was also an issue that arose from the papers. Because of this fact, all results are presented with the risk of bias figures alongside the forest plots. Before reporting the results of the meta-analyses performed, a short definition of intestinal failure and short bowel syndrome is provided. The reason for this is that many conditions
that will be described include various forms of intestinal failure and the necessary nomenclature is imperative.

Intestinal failure occurs when there is reduced intestinal absorption so that intravenous macronutrient and/or water and electrolyte supplements are needed to maintain health and/or growth (Pironi et al., 2016). If untreated, undernutrition and/or dehydration result. Nutrient/fluid requirements determine whether intestinal failure is termed severe, moderate, or mild. Severe is when parenteral, moderate when enteral, and mild when oral nutritional fluid supplements are needed (Nightingale and Woodward, 2006). A patient may change, due to compensatory mechanisms (for example functional adaptation), from having severe to mild intestinal failure with time. Intestinal failure is classified into three types: Type I, acute, short-term, and often self-limiting condition; Type II, prolonged acute condition, often in metabolically unstable patients, requiring complex multidisciplinary care and intravenous supplementation over periods of weeks or months; and Type III, chronic condition, in metabolically stable patients, requiring intravenous supplementation over months or years (Pironi et al., 2016). It may be reversible or irreversible, acute or chronic and encompasses five pathophysiological mechanisms (short bowel syndrome, intestinal fistula, intestinal dysmotility, mechanical obstruction, extensive small bowel mucosal disease) from a wide variety of underlying medical and surgical conditions (mesenteric ischemia, Crohn’s disease, coeliac disease, immunodeficiency, radiation enteritis, surgical complications, familial polyposis, volvulus, intestinal malformation, necrotizing enterocolitis, primary chronic intestinal pseudo-obstruction, scleroderma, Hirschsprung’s disease, immunodeficiency and other conditions). These patients may be most appropriately managed in a centre with special interest and expertise in intestinal failure (Nightingale, 2001).

Normal human small intestinal length, measured from the duodenojejunal flexure at autopsy or surgery, varies from about 275 cm to 850 cm, and tends to be shorter in women. After intestinal resection it is important to refer to the remaining length of small intestine measured at surgery or with an opisometer rather than to the amount removed (Nightingale, 2001). In general, nutritional/fluid supplements are likely to be needed if less than 200 cm of small bowel remains.
There are three main types of patient with a short bowel: those who have had a jejunoileal resection and a jejunocolic anastomosis (*jejunum-colon*); those who have had a predominantly jejunal resection, and have more than 10 cm of terminal ileum and the colon remaining (*jejunum-ileum*); and those who have had a jejunoileal resection, colectomy, and formation of a stoma (*jejunostomy*). Jejunum-ileum patients are uncommon and rarely have problems of undernutrition and therefore do not often need nutritional support. When jejunum-ileum patients are seen, with undernutrition or severe diarrhoea after eating/drinking, they are managed in the same way as jejunum-colon patients. Jejunum-colon and jejunostomy patients are most commonly encountered (Nightingale *et al.*, 1992).

The most common reasons for a short bowel in adults are Crohn’s disease, superior mesenteric artery thrombosis, and irradiation damage (Table 2.7) (Nightingale and Woodward, 2006; Pironi *et al.*, 2016; Pironi *et al.*, 2018). Jejunum-colon patients often appear well after their resection except for diarrhoea/steatorrhea, but in the following months may lose weight and become severely undernourished. Patients with a jejunostomy have problems of dehydration immediately after surgery due to large stomal water and sodium losses. This jejunal output is greatest after food and drink consumption.

Next, the specific conditions where plasma citrulline levels have been described as indicating enterocyte function are reported alongside the meta-analyses’ results.

### 2.3.1 Necrotizing Enterocolitis (NEC)

Necrotizing enterocolitis is a medical condition primarily seen in premature infants, where portions of the bowel undergo necrosis (tissue death) (Lin and Stoll, 2006). It occurs postnatally and is the second most common cause of mortality in premature infants. The combination of a genetic predisposition, intestinal immaturity, and an imbalance in microvascular tone, accompanied by a strong likelihood of abnormal microbial colonization in the intestine and a highly immunoreactive intestinal mucosa, leads to a confluence of predisposing factors (Neu and Walker, 2011). The most typical initial signs and symptoms of necrotizing enterocolitis in a preterm infant include feeding intolerance, abdominal distention and bloody stools after 8 to 10 days of age (Lin and Stoll, 2006; Neu and Walker, 2011). Treatment consists primarily of supportive care with bowel rest and any surgery performed may lead up to short bowel syndrome (Lin and Stoll, 2006).
In this context, four studies have assessed citrulline levels in patients with necrotizing enterocolitis (Table 2.1). Risk of bias was low in most. The MD indicated a significant decrease in citrulline levels by -7.8 μmol/L (95% CI [-14.7, 0.9]; \( I^2 = 97\% \)) compared to control, which indicated a strong decrease when the SMD was analysed -1.44 (95% CI [-2.80, -0.07]; \( I^2 = 96\% \)) (Figure 2.4). Celik et al. (2013) described that the area under the receiver operating characteristic (ROC) curve for citrulline to differentiate NEC from controls was 0.88 (95% CI, 0.77-0.99) and cut-off level of citrulline was 13.15 μmol/l with a sensitivity of 80% and a specificity of 82% but no association with duration of parenteral nutrition was noted. Similarly, Ioannou et al. (2012) noted that the area under the ROC curve for plasma citrulline to discriminate neonates with NEC from control neonates was 0.86 (95% CI, 0.77-0.96). The citrulline level that maximized the test’s sensitivity and specificity was 17.75 μmol/L, with a sensitivity of 76% and a specificity of 87%.

2.3.2 Intestinal Transplantation

Intestinal transplantation or small bowel transplantation is the surgical replacement of the small intestine for cases of permanent intestinal failure. While intestinal failure can often be treated with alternative therapies such as parenteral nutrition (PN), complications such as intestinal failure associated liver disease and short bowel syndrome may make transplantation the only viable option (Nightingale, 2001; Goulet and Sauvat, 2006). The rarest type of organ transplantation performed, intestinal transplantation is becoming increasingly prevalent as a therapeutic option due to improvements in immunosuppressive regiments, surgical technique, and the clinical management of pre and post-transplant patients (Fishbein, 2009).

Measurement of citrulline levels has sparked the interest of specialists in intestinal transplantation as a possible indicator of rejection – and this is completely reasonable, considering the consequences that may arise. Thirteen studies were identified in the literature search and two groups have published quite extensively in the field: the University of Miami School of Medicine (Pappas et al., 2001; 2002; 2004a; 2004b; Yu et al., 2005; David et al., 2006; 2007; 2008; Ruiz et al., 2010; Hibi et al., 2012) and the Mount Sinai School of Medicine (Gondolesi et al., 2002; 2004; 2006) (Table 2.2).
These studies’ focus is the ability of citrulline levels to predict the grade of acute cellular rejection and the cut-off value of citrulline levels that yield a high possibility of acute cellular rejection. There was presence of detection bias and confounding because not all studies assessed possible confounders of associations. Also, there is a strong possibility of reporting bias and attrition bias, because many papers are published in Transplantation Proceedings, which publishes short reports from transplant centres (Figure 2.5). The initial studies by the Miami group described a moderate negative correlation of citrulline levels with rejection (Pappas et al. (2001) reported $CC = -0.59$) but in the recent studies by Ruiz et al. (2010) and Hibi et al. (2012), which include up to around 10,000 plasma citrulline samples, correlation reaches up to a strong $-0.977$ with acute cellular rejection. The correlation coefficients are shown in Figure 2.5 without meta-analysis due to severe heterogeneity.

Two other trends were noted in the transplantation articles: first, citrulline appears to normalize after a certain amount time post transplantation and this is a significant factor against rejection (Pappas et al., 2002; Gondolesi et al., 2004); Pappas et al. (2004a); Pappas et al. (2004b); secondly, the cut-off value of citrulline that could very strongly predict rejection. The Miami group have described in two papers that citrulline levels have a very high negative predictive value for moderate or severe acute rejection in these patients (negative predictive value = 99% with cut-off level 13 μmol/L; sensitivity is 96.4% with particularly high specificity in adult patients) (David et al., 2007; David et al., 2008; Hibi et al., 2012) but the Mount Sinai Group did not find that citrulline had satisfactory diagnostic accuracy to discern rejection (Gondolesi et al., 2006).

In conclusion, results suggest the use of citrulline as a measure of graft rejection in intestinal transplantation. However, cautious interpretation is necessary due to sequential measurements (in contrast to single measurements in other conditions) and presence of heterogeneity between studies. Gut function and physiology can be affected by time after surgery, renal function, graft pathology, infection/sepsis, donor and patient anthropometrics post intestinal transplant (Gondolesi et al., 2002; Gondolesi et al., 2004; Gondolesi et al., 2006) with normalization of citrulline not always occurring after treatment of graft versus host disease (Pappas et al., 2004a).
Future research should focus on investigating the predictive power of plasma citrulline measurements against other biomarkers.

2.3.3 *Short Bowel Syndrome*

Citrulline’s unique metabolism which involves production at the intestinal enterocyte has prompted suggestions that plasma citrulline level could be a reliable marker of gut function (Crenn, 2008; Crenn *et al*., 2008). This led to a hypothesis that citrulline may be a *conditionally* essential amino acid in short bowel syndrome, even if it is not incorporated into proteins. From the literature review, 35 papers and abstracts were identified which included eventually 26 studies (Table 2.3). Quality assessment showed possibilities of reporting, attrition and detection bias (Figures 2.2 and 2.3). Several outcomes were analysed in the short bowel syndrome group of studies.

2.3.3.1 *Citrulline and Residual Small Bowel Length*

Twenty one studies were analysed and the random effects analysis of correlation coefficients produced a pooled effect of 0.68 (95% CI 0.37-0.85, range 0.26-0.99), which indicates a strong correlation. In addition there was evidence of publication bias (funnel plot asymmetry, Egger’s test $p = 0.001$ but Begg’s test $p = 0.156$, Failsafe number = 5286) and high heterogeneity ($Q = 764.98$, df = 20, $p < 0.001$; $I^2 = 97\%$, $\tau^2 = 0.99$). See Figure 2.6 for the forest plot of this outcome and Figure 2.7 for the funnel plot.

Subgroup analyses were performed to explore heterogeneity (Figure 2.8):

1. When correlations were analysed by country, Spain, the USA and Italy had reduced $I^2$ (0%, 4% and 56.4% respectively). In the French group of papers, heterogeneity remained high ($I^2 = 98.6\%$).

2. When correlations were analysed by continent, America and Asia had no heterogeneity ($I^2$ was 4% and 0% respectively) while heterogeneity remained high in Europe ($I^2 = 97.9\%$).

3. When correlations were analysed by population, the adults group had still high heterogeneity $I^2 = 98.2\%$ while the paediatric group had less heterogeneity $I^2 = 62\%$. 
4. When correlations were analysed by citrulline measurement method, heterogeneity remained high in all groups (HPLC, IEC and mass spectrometry).

Finally, we performed metaregression with four continuous variables that could possibly affect this association: male percentage, mean age, mean BMI, mean citrulline concentration and mean small bowel length. No variable had significant effect on the mean correlation and the model was not significant (Table 2.8).

Speculating on the causes of heterogeneity, most likely the heterogeneity in France is due to measurement methods between institutions and unlikely to be ethnic anatomical variations between different countries. Although both children and adult studies had heterogeneity, this was higher in adults. Adult studies were predominantly performed in European countries, whilst most studies from the US were performed in children.

2.3.3.2 Citrulline between SBS Patients and Healthy Controls

Twelve studies were analysed and the random effects model showed that citrulline levels were decreased by -12 μmol/L (95% CI [-16.3, -7.7]) (SMD -1.34 (95% CI [-1.77, -0.91])); there was heterogeneity (MD: $Q = 134.69$, df = 11, $p < 0.001$; $I^2 = 92\%$, $\tau^2 = 49.75$; SMD: $Q = 56.39$, df = 11, $p < 0.001$; $I^2 = 80\%$, $\tau^2 = 0.44$) but no publication bias (symmetric funnel plot, Egger’s test $p = 0.606$, Begg’s test $p = 0.537$, Failsafe $N = 853$) (Figure 2.4 and Figure 2.9B).

2.3.3.3 Citrulline Levels in PN Dependent vs PN Independent Patients

Twelve studies were analysed comparing levels of citrulline in patients who needed parenteral nutrition against patients who were weaned off PN. The random effects model showed that citrulline levels were decreased by -13.3 μmol/L (95% CI [-17.6, -9.0]) (SMD -1.58, 95% CI [-2.09, -1.08]); there was heterogeneity (MD: $Q = 97.28$, df = 11, $p < 0.001$; $I^2 = 89\%$, $\tau^2 = 46.89$; SMD: $Q = 52.10$, df = 11, $p < 0.001$; $I^2 = 79\%$, $\tau^2 = 0.57$) but no publication bias (symmetric funnel plot, Egger’s test $p = 0.174$, Begg’s test $p = 0.451$, Failsafe $N = 753$) (Figure 2.10A and Figure 2.9D).

Sixteen studies described diagnostic accuracy results and these were meta-analysed with the hierarchical summary receiver operating characteristic (ROC) model by Harbord et al. (2007). The details of each study are shown in Table 2.9, which shows individual sensitivities, specificities, citrulline cut-off levels and values for
true positives, false positives, false negatives and true negatives. The results are shown in Table 2.10, forest plots for sensitivity and specificity are shown in Figure 2.11 while the sROC curve is shown in Figure 2.12. Overall, citrulline levels have a sensitivity of 82.5% and specificity 82%. Since thirteen studies compared citrulline levels at a cut-off level of 20 μmol/L to discern among short bowel syndrome patients who needed PN or not, the meta-analysed sensitivity and specificity reflect mostly that. Any heterogeneity is present due to different cut-off levels and comparison groups. Healthy patients with normal intestinal mucosa function and normal renal function have a citrulline level between 30 and 50 μmol/L with a median of 40 μmol/L (Rabier and Kamoun, 1995; Curis et al., 2005). Although this range for plasma citrulline levels mainly comes from studies in Western Europe and North America, a Chinese study on 33 healthy Chinese subjects found a mean plasma citrulline level of 16.87 ± 5.97 μmol/L (range 19-54) measured with HPLC (Liu et al., 2004). This finding is very interesting, becomes it creates a paradox in the multiple findings of the high diagnostic accuracy of citrulline level at 20 μmol/L.

2.3.3.4 Teduglutide and Citrulline Levels

There have been four studies studying the effect of teduglutide – a GLP-2 analogue which acts as a growth factor in patients with short bowel syndrome – on citrulline levels (Gilroy et al., 2009; Jeppesen et al., 2009; Buchman et al., 2010; 2011; 2012; Naimi et al., 2013; Seidner et al., 2015). Three studies compared citrulline levels in patients who received teduglutide against patients who received placebo in Crohn’s disease (Buchman et al., 2010) and short bowel syndrome (Gilroy et al., 2009; Jeppesen et al., 2009; 2011; 2012; Naimi et al., 2013; Seidner et al., 2015). The random effects model showed that citrulline levels in teduglutide versus placebo were increased by 12.4 μmol/L (95% CI [5.5, 19.3]) (SMD 1.02, 95% CI [0.47, 1.58]) and there was heterogeneity (MD: $Q = 13.13$, df = 2, $p = 0.001$; $I^2 = 85\%$, $\tau^2 = 31.01$; SMD: $Q = 7.41$, df = 2, $p = 0.02$; $I^2 = 73\%$, $\tau^2 = 0.18$) (Figure 2.10B). Four studies provided citrulline levels of patients who received teduglutide at the end of treatment compare to their baseline (Gilroy et al., 2009; Jeppesen et al., 2009; Buchman et al., 2010; 2011; 2012; Naimi et al., 2013; Seidner et al., 2015). The random effects model showed that citrulline levels in teduglutide at end of treatment versus baseline were increased by 15.3 μmol/L (95% CI [12.5, 18.2])
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(SMD 1.21, 95% CI [1.00, 1.43]); there was no heterogeneity (MD: $Q = 3.57$, df = 3, $p = 0.31$; $I^2 = 16\%$, $\tau^2 = 1.41$; SMD: $Q = 2.05$, df = 3, $p = 0.56$; $I^2 = 0\%$, $\tau^2 = 0.00$) (Figure 2.10C).

Historically, short bowel syndrome was the first pathological situation to be studied in conjunction with citrulline levels due to its innate experimental nature, created by the removal of a significant amount of the anatomical and functional mass of intestine. Since Crenn et al. (2000) published their study, there have been at many more studies performed in adults (see Table 2.3). All of these studies have found a strong and significant positive correlation, with CCs ranging from 0.26 to 0.99, between postabsorptive plasma citrulline concentration and remnant small bowel length. It appears that citrulline concentration reflects the overall small bowel function, including small bowel excluded from the digestive circuit; hence, determination of citrulline concentration can be used preoperatively as a reliable biomarker of the probability of parenteral nutrition weaning at a 20 µmol/L threshold (Crenn et al., 2000).

2.3.4 Enteropathies

2.3.4.1 Villous Atrophy Syndrome

In chronic villous atrophy (e.g., coeliac disease, tropical sprue, and various infectious enteritides), citrulline is decreased (less than 20 µmol/L) in patients with proximal destructive lesions of villous architecture and severely decreased in patients with extensive (proximal and distal) impairment of intestinal mucosa (Curis et al., 2007).

The first study to associate citrulline levels with enterocyte damage from coeliac disease was by Crenn et al. (2003). Their main results showed that plasma citrulline concentration was lower ($p < 0.001$) in patients with villous atrophy (24 ± 13 µmol/L) than in healthy subjects (40 ± 10 µmol/L) and patients with anorexia nervosa (39 ± 9 µmol/L). Three thresholds were individualized: less than 10 µmol/L for patients with diffuse total villous atrophy ($n = 10$), 10-20 µmol/L for patients with proximal-only total villous atrophy ($n = 12$), and 20-30 µmol/L for patients with partial villous atrophy ($n = 10$). Plasma citrulline concentration was correlated to the severity and extent of villous atrophy ($r = 0.81; p < 0.001$) while ROC curves indicated that plasma citrulline concentration was the best biological variable to
predict villous atrophy. In these patients, a citrulline value below 10 µmol/L is highly predictive of the need for at least one period of PN. The patients who received PN had invasive small bowel lymphoma, refractory sprue with cryptic lymphoma, and extensive small intestinal CD4 T-cell infiltration all with severe malabsorption and diffuse destructive mucosal lesions.

Similar results were recently reported in patients with human immunodeficiency virus enteropathy or severe intestinal infectious disease (Crenn et al., 2009a). In this situation, plasma citrulline levels can be considered as an indicator of evolution of the intestinal disease. Patients with only mild enterocyte involvement, i.e., partial proximal villous atrophy, have a normal or moderately decrease in citrulline concentration. The results by Crenn et al. (2009a) were verified by Papadia et al. (2010). In their study, postabsorptive fasting serum citrulline was measured in 150 tropical enteropathy patients, 44 of whom had HIV infection, using reverse phase HPLC. Absorptive capacity and permeability were measured after intrajejunal instillation of 5 g lactulose, 1 g L-rhamnose, 0.5 g D-xylose, 0.2 g 3-O methyl D-glucose with assay by thin-layer chromatography. In human immunodeficiency virus (HIV) positive patients, the median serum citrulline was significantly lower (median 19 µmol/L) than in HIV negative patients (median 27 µmol/L; p < 0.001).

There were statistically significant correlations (p < 0.005) between citrulline and crypt depth; villous height/crypt depth ratio; Shenk-Klipstein score; and xylose absorption, only in the HIV positive. Thus, serum citrulline concentration appears to be a quantitative biomarker of small bowel mass integrity in HIV positive enteropathy.

Eleven studies were used in meta-analyses in this category which included cases that had coeliac disease or other enteropathy (tropical, HIV etc.) (Table 2.4). Meta-analyses firstly compared citrulline levels in diseased patients vs controls, then those who had received gluten free diet (GFD) compare to those who hadn’t, and finally association of citrulline levels with disease severity. Severity of disease was categorised broadly and included either histological diagnoses, worsening symptoms or any other metric reported by authors which indicated severity of the enteropathy. Severity is to be considered as a scale by which higher values indicate more severe disease and lower values indicate less severe disease. The random effects model showed that citrulline levels in coeliac disease/enteropathy patients
compared to control were decreased by -9.5 μmol/L (95% CI [-12.9, -6.1]) (SMD -0.99, 95% CI [-1.30, -0.67]); there was heterogeneity (MD: $Q = 77.52$, df = 9, $p < 0.001$; $I^2 = 88\%$, $\tau^2 = 23.08$; SMD: $Q = 41.51$, df = 9, $p < 0.001$; $I^2 = 78\%$, $\tau^2 = 0.19$) but no publication bias (symmetric funnel plot, Egger’s test $p = 0.247$, Begg’s test $p = 0.283$) (Figure 2.4 and Figure 2.9C). Citrulline levels were compared in coeliac disease patients who had received GFD vs those who hadn’t in five studies (Crenn et al., 2003; Hozyasz et al., 2006; Miceli et al., 2008; Blasco Alonso et al., 2011; Ioannou et al., 2011); the random effects model showed that citrulline levels were decreased by -8.2 μmol/L (95% CI [-10.4, -5.9]) (SMD -1.08, 95% CI [-1.42, -0.75]) in those patients who hadn’t received GFD; there was no heterogeneity (MD: $Q = 4.08$, df = 4, $p = 0.39$; $I^2 = 2\%$, $\tau^2 = 0.15$; SMD: $Q = 2.47$, df = 4, $p = 0.65$; $I^2 = 0\%$, $\tau^2 = 0.00$) (Figure 2.10D).

### 2.3.4.2 Crohn’s Disease

Papadia et al. (2007) first studied the levels of plasma citrulline in patients with Crohn’s disease (CD). They studied 55 CD patients according to diagnosis, small bowel length, and degree of bowel inflammation. Their results were very interesting in the fact that they showed a positive relationship between citrulline and small bowel length for lower citrulline values ($p < 0.001$), but this relationship tailed off for higher citrulline values. Citrulline concentrations correlated with small bowel absorptive capacity for carbohydrate, while the presence of intestinal inflammation didn’t affect plasma citrulline concentrations. (Buchman et al., 2010) investigated CD patients who had received teduglutide to induce remission and measured plasma citrulline as surrogate marker of mucosal healing. Their results suggest a potential link between improved mucosal healing and increase in plasma citrulline, indicating that citrulline could function as a surrogate marker of intestinal absorption.

Citrulline levels were compared between Crohn’s disease patients and controls in two studies (Papadia et al., 2007; Diamanti et al., 2011a). The random effects model showed that citrulline levels in patients vs controls was decreased by -9.7 μmol/L (95% CI [-12.6, -6.7]) (SMD -1.19, 95% CI [-1.63, -0.75]); there was no heterogeneity (MD: $Q = 0.02$, df = 1, $p = 0.90$; $I^2 = 0\%$, $\tau^2 = 0.00$; SMD: $Q = 0.00$, df = 1, $p = 0.99$; $I^2 = 0\%$, $\tau^2 = 0.00$) (Figure 2.4).
2.3.4.3 Acute Mucosal Enteropathy and Cancer Treatments

Acute mucosal enteropathy can cause a significant loss of enterocytes, as experienced clinically by secondary lactose intolerance. Plasma citrulline concentrations are reduced in these situations, for example, in adenovirus enteritis and in all infectious intestinal diseases with high cytopathic effect (Gondolesi et al., 2006). The normalization of citrullinemia is often rapid, after 1-3 weeks. Chemotherapy in haematology (e.g. as part of conditioning regimens for bone marrow transplants) and oncology induces a decrease in citrulline levels, which relates with the known cytokinetic renewal of intestinal mucosa with a nadir 5-8 days after treatment initiation (Blijlevens et al., 2004; Lutgens and Lambin, 2007). Citrullinemia is more sensitive and more specific than the sugar-based permeability test for detecting chemotherapy-induced gut damage in patients with haematological malignancies (Blijlevens et al., 2004). After bone marrow transplantation, the decrease in citrullinemia appears to be a risk factor for infections (predominantly septicaemia and intestinal infections) (Blijlevens et al., 2004). This suggests that, during antineoplastic therapies, citrullinemia could be used to monitor the intestinal mucosa toxicity. Mucositis and epitheliitis can be treated or prevented in part by keratinocyte growth factor. In a mouse model, recombinant human keratinocyte growth factor treatment allowed maintenance of the citrulline level at a normal value during chemotherapy (Lutgens et al., 2003; Lutgens et al., 2004). Acute radiation enteritis induced by total body irradiation or fractioned localized irradiation can be monitored by citrullinemia that correlates to dose received and volume of bowel in the field of radiation (Lutgens et al., 2003; Lutgens et al., 2004; Vaanleer et al., 2005).

Fourteen studies were used in meta-analysis in this category which included cases of patients who had received chemoradiation for bone marrow transplants, cancer or other malignant disorder (Table 2.5). The most common theme that these papers employ is the use of citrulline levels to diagnose and monitor gastrointestinal toxicity related to treatment or mucositis. Generally, most papers described that:

1. Citrulline decreases in the initial phase of treatment and then increases while the initial toxicity related to treatment seem to reside.

2. Gastrointestinal toxicity involves diarrhoea, pain, vomiting and mucositis.
3. Citrulline decrease is related to higher doses of chemoradiation treatment and is usually inversely correlated with severity of gastrointestinal toxicity. Meta-analysis was performed on this outcome since 14 studies were recognised.

The random effects model showed that citrulline levels were negatively correlated with severity of gastrointestinal toxicity with a moderate correlation of -0.41 (95% CI [-0.52, -0.29]) (Figure 2.13); there was no heterogeneity ($Q = 24.4$, df = 13, $p = 0.03$; $I^2 = 47\%$, $\tau^2 = 0.03$) and no publication bias (symmetric funnel plot, Egger’s test $p = 0.009$, Begg’s test $p = 0.102$) (Figure 2.9F).

2.3.5 Critical Illness Patients

Twenty five studies were diagnosed investigating citrulline levels in patients with critical illness (Table 2.6). The majority of studies involve patients in intensive care unit (ICU) settings which attempt to correlate citrulline levels with severity of condition or other sepsis markers. No meta-analyses were performed on these studies due to different study designs with heterogeneous patient populations not allowing data extraction. The following comments can be made:

1. Citrulline appears decreased in most studies and is related to critical illness and markers of sepsis or inflammation.

2. This decrease in citrulline doesn’t necessarily mean that there is intestinal dysfunction since in inflammatory responses as severe as critical illness, nitric oxide and arginine are depleted through inflammatory pathways hence leading to the reduction of citrulline (Luiking et al., 2009). This is also corroborated by the fact that citrulline levels increase once critical condition is overcome.

3. Citrulline seems to act as a negative inflammatory marker as is albumin in inflammatory or septic conditions. Its use as a marker of intestinal compromise should be cautious.

2.3.6 Citrulline Levels: An Overall Assessment

2.3.6.1 Diagnostic Accuracy

In this section a meta-analysis of citrulline’s diagnostic accuracy is performed in all patient groups (26 studies) with hierarchical summary ROC analysis (Harbord et al., 2007). The overall sensitivity of citrulline levels appeared to be satisfactory.
80% (95% CI 69%-87%) (Figure 2.11; Table 2.10); specificity was 84% (95% CI 77%-89%) (Figure 2.11); and the diagnostic odds ratio was 20.03. The sROC indicated overall satisfactory diagnostic accuracy of citrulline levels (Figure 2.14).

2.3.6.2 Citrulline Levels in Diseased Patients versus Controls

The random effects model showed that citrulline levels in patients versus controls (30 studies) was decreased by -11.2 μmol/L (95% CI [-13.8, -8.6]) (SMD -0.53, 95% CI [-0.69, -0.36]); there was no heterogeneity (MD: $Q = 56.07$, df = 29, $p = 0.002$; $I^2 = 48.3\%$, $\tau^2 = 23.77$; SMD: $Q = 92.64$, df = 29, $p < 0.001$; $I^2 = 68.7\%$, $\tau^2 = 0.12$) (Figure 2.4). No publication bias was observed (symmetric funnel plot, Egger’s test $p = 0.969$, Begg’s test $p = 0.986$) (Figure 2.9A).

2.3.6.3 Citrulline Levels as a Marker of Intestinal Disease Severity

Citrulline levels were described in association with disease severity in 28 studies. The random effects model showed that citrulline levels were negatively correlated with severity of disease with a moderate correlation of -0.56 (95% CI [-0.70, -0.37]) (Figure 2.13); there was heterogeneity ($Q = 582.68$, df = 27, $p < 0.001$; $I^2 = 95\%$, $r^2 = 0.38$) but no publication bias (symmetric funnel plot, Egger’s test $p = 0.356$, Begg’s test $p = 0.722$) (Figure 2.9E). Interestingly, only in Crohn’s disease, citrulline is not associated with disease severity (Figure 2.13).

2.3.6.4 Citrulline and Absorptive Function

Fourteen studies reported an association of citrulline levels with the level of enteral absorption. Absorption was assessed with the D-xylose absorption test, oral or enteral nutrition tolerance, and nutrient absorption tests with bomb calorimetry and measuring oral/enteral intake in comparison to faecal and other loses. The random effects model showed that citrulline levels were positively correlated with enteral absorption with a moderate correlation of 0.47 (95% CI [0.27, 0.62]) (Figure 2.15) but there was heterogeneity ($Q = 59.26$, df = 13, $p < 0.001$; $I^2 = 78\%$, $r^2 = 0.13$).

Overall, there are fewer results concerning the relationship between citrulline and absorptive function. Crenn et al. (2000) suggested a positive relationship between citrullinemia and percentage of fat ($r = 0.53$) and nitrogen ($r = 0.47$) absorbed. Rhoads et al. (2005) determined in children that plasma citrulline concentration enables estimation of the percentage of enteral calories that a short gut can tolerate
without diarrhoea. Luo et al. (2007) however, did not find any significant relationship between citrulline and macronutrient (nitrogen, fat, carbohydrate, calories), fluid, and electrolyte (sodium, potassium, phosphorus, magnesium) absorption expressed in percentage of amount ingested in adult short bowel syndrome patients. Thus, it is likely that plasma citrulline concentrations cannot reflect equally the various aspects of gut function. Citrulline reflects the integrity of the intestinal epithelial cells with a predominant site of production at the proximal jejunum, whereas absorption is a complex integrated function related to small bowel mucosa, biliopancreatic secretions, digestive motility, gut lumen, and colonic absorption (Curis et al., 2007; Papadia et al., 2007; Peters et al., 2008b). In addition, the absorption process is variable in capacity and location according to the nutrients considered. Hence, citrulline concentration might be an indicator of the functional enterocyte metabolic mass but less exclusively of the digestive and absorptive function.

2.4 Summary of Findings, Discussion, and Conclusion

The present study is the first meta-analysis on the association of citrulline with gut function. Although citrulline appears to be a strong marker of enterocyte mass, its correlation with intestinal absorption is weaker. A systematic review of studies was performed along with a statistical meta-analysis. Overall plasma citrulline measurements have been used in necrotizing enterocolitis, intestinal transplantation, short bowel syndrome, enteropathies (villous atrophy syndromes, Crohn’s disease, and mucositis) and critical illness patients. Citrulline was strongly positively correlated with enterocyte mass. This correlation was clinically significant in short bowel syndrome. In other conditions where short bowel is not an issue, there is a decrease in average citrulline levels compared to healthy controls and citrulline decrease can be correlated to the degree of disease severity apart from Crohn’s disease. Its interpretation should be cautious because its diagnostic accuracy is satisfactory but doesn’t exclude absolutely false negative or false positive cases. Factors that influence interpretation are lower population values, combination of intestinal disorders, possible bowel adaptation, measurement timing, inflammation and extent of disease. Citrulline was correlated positively with enteral absorption, albeit moderately. Although a higher plasma citrulline level suggests a more absorptive gut, this interpretation needs to take into consideration other factors such
specific macronutrients, condition examined, extent of mucosal disease etc. A cut-off citrulline level at 20 μmol/L has sensitivity and specificity of 80% in all conditions. Limitations of the meta-analysis was presence of heterogeneity and the possibility of publication bias, detection bias and confounding bias.

Hence, returning to the questions posed in the introduction:

1) Is citrulline a successful indicator of enterocyte mass?

Yes, citrulline was strongly positively correlated with enterocyte mass. This correlation was clinically significant in short bowel syndrome. In other conditions where short bowel isn’t an issue, there is a decrease in average citrulline levels compared to healthy controls and citrulline decrease can be correlated to the degree of disease severity apart from Crohn’s disease. Its interpretation should be cautious because its diagnostic accuracy is satisfactory but doesn’t exclude absolutely false negative or false positive cases. Factors that influence interpretation are lower population values, combination of intestinal disorders, possible bowel adaptation, measurement timing, inflammation and extent of disease.

2) Is citrulline a successful marker of enterocyte absorption?

Yes, citrulline was correlated positively with enteral absorption, albeit moderately. Although a higher plasma citrulline level suggests a more absorptive gut, this interpretation needs to take into consideration other factors such specific macronutrients, condition examined, extent of mucosal disease etc.

3) What clinical conditions has citrulline been utilized in as a marker?

Citrulline has been examined in short bowel syndrome, intestinal transplant, coeliac disease, Crohn’s disease, other enteropathies, necrotizing enterocolitis and critical illness. In all conditions except critical illness, the gut is compromised and in this case a decrease in citrulline seems to be attributed mainly to this. However, in critical illness the interpretation of a low citrulline as a marker of intestinal dysfunction should be treated with caution – in a similar manner that a low albumin in a critical ill patients needs to be cautiously interpreted as malnutrition. The availability of nitric oxide and arginine during septic and inflammatory states is decreased hence decreasing citrulline and in this context citrulline could be a negative inflammatory marker – without of course excluding the possibility of acute illness enteropathy. The mechanism of the enteropathy in this condition would be a
combination of inflammatory and possibly infective triggers leading to colitis or malabsorption and related sequelae.

4) Can a new theory regarding citrulline’s utility be suggested?

I am going to advocate that this can be done fairly confidently. The synthesis of literature has shown that citrulline concentration is decreased compared to controls in circumstances of intestinal compromise; it has a sensitivity and specificity of 80% in all conditions; it is negatively correlated with disease severity in intestinal enteropathies (except Crohn’s disease); it is positively correlated with small bowel length in short bowel syndrome; and it is moderately correlated with enteral absorption in various conditions. I would like to introduce the term likely acute gut failure (similar to intestinal insufficiency) at this point and hence propose that interpretation of citrulline levels leads to diagnoses of possible acute gut failure.

Limitations of the present meta-analysis stem from various sources of heterogeneity and possibility of publication bias, detection bias and confounding bias. It was a pattern in the present review that many studies didn’t analyse confounding factors such as other amino acids, renal function (citrulline’s pathways involve a renal component) and inflammatory state. Hence, for future studies it is suggested that nitric oxide, full amino acid profiles (or at least arginine, glutamine and ornithine) are measured when citrulline is measured, renal function and inflammatory state.

Heterogeneity was a frequent observation in this study and the source was not always found. One needs to take into account different measuring methods for citrulline (IEC, HPLC, TMS, ELISA) which were used, sample preparation, population parameters, different scales for documenting disease severity, absorption, small bowel length and so on. Each biological method and clinical assessment scale has inherent variability which is added up when a meta-analysis is performed and this leads to heterogeneity. The random effects models performed takes heterogeneity into account and thus the results presented are less burdened from it when interpreted – meaning that when significant results are reported with meta-analysis despite increased heterogeneity, this is indicative of a truly strong effect.

Another interesting addition of this study is the study of diagnostic accuracy for citrulline. There were various thresholds for discerning a high from low citrulline level but the level of 20 μmol/L seems to be most prevalent. It also indicates that
under these levels, clinical alarms need to be triggered – taking into account the confounding factors of course.

In the next chapter, the clinical experiments performed at UCL for investigating citrulline physiology in a pharmacodynamics study are described.
### 2.5 Tables

#### Table 2.1  Necrotizing enterocolitis studies.

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<tr>
<th>No</th>
<th>Authors</th>
<th>Settings (sample and design)</th>
<th>Main Results</th>
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</table>
| 1  | Becker et al. (2000)     | • Aim: to determine whether premature infants who have necrotizing enterocolitis have deficiencies in glutamine and arginine  
• 4-month prospective cohort study of serum amino acid and urea levels in premature infants  
• Serum amino acid and urea levels were measured by high-pressure liquid chromatography and enzymatic methods  
• Control (n = 32), necrotizing enterocolitis (n = 13) (comparable for birth weight, gestational age, and Apgar scores)                                                                                     | • Days 7, 14, 21: Median values of glutamine: 37 % to 57 % lower in the necrotizing enterocolitis group compared to control group (p < 0.05)  
• Days 7 and 14: Median values of arginine, glutamine, alanine, lysine, ornithine, and threonine were decreased by 36 % to 67 % (p < 0.05) in the necrotizing enterocolitis group  
• Citrulline levels were decreased in the necrotizing enterocolitis group compared to control (p < 0.05)     |
| 2  | Ioannou et al. (2012)    | • Plasma citrulline levels were measured prospectively in 17 preterm neonates with necrotizing enterocolitis stage II during the entire course of the disease  
• Serial citrulline determinations in 24 healthy preterm neonates on 2, 7, 14, 21 and 28 days of life, served as reference values                                                                                   | • In healthy preterm neonates plasma citrulline levels showed a progressive increase in relation to age  
• In neonates presenting with necrotizing enterocolitis, mean citrulline levels were significantly lower as compared to controls’ citrulline levels (day of life 7: 16.85 ± 4.2 vs 20.5 ± 4.5 μmol/L, p < 0.05; day of life 14: 18 ± 4.2 vs 23.5 ± 4.3 μmol/L, p < 0.01; day of life 21: 17 ± 2.5 vs 30 ± 5.7 μmol/L, p < 0.01)  
• Optimal citrulline cut-off distinguishing necrotizing enterocolitis patient from controls: 17.75 μmol/L (sensitivity 76%, specificity 87%)  
• Plasma citrulline at presentation correlated inversely with the duration of parenteral nutrition (r=-0.49, p<0.05)     |
| 3  | Celik et al. (2013)      | • Plasma citrulline levels of neonates with a gestational age less than 32 weeks and ≤ 1500 g who developed necrotizing enterocolitis stage II/III were measured by high-performance liquid chromatography  
• 36 preterm infants including 20 with necrotizing enterocolitis and 16 controls                                                                                                                           | • Median citrulline levels of necrotizing enterocolitis and control groups were 8.6 and 20.18 μmol/L (p < 0.05), and cut-off level of citrulline was 13.15 μmol/L with a sensitivity of 80% and a specificity of 82%  
• Median arginine levels of necrotizing enterocolitis and control groups were 22.02 and 39.89 μmol/L (p < 0.05), and cut-off level of arginine was 28.52 μmol/L with a sensitivity of 70% and a specificity of 75%  
• Blood sampling day, gender, and parenteral or enteral nutrition did not affect amino acid levels                                                                                                               |
| 4  | Englund et al. (2014)    | • Aim: To determine whether citrulline concentrations measured in neonatal dried blood spots could predict necrotizing enterocolitis  
• National Danish registries were retrospectively searched to identify 361 babies born between 2003 and 2009, diagnosed with necrotizing enterocolitis and a valid citrulline concentration  
• Control group: 1083 healthy new-borns (three controls for every new-born with necrotizing enterocolitis, matched for birthweight and gestational age)                                                                                      | • Neonatal dried blood spots were collected between 2 and 21 days of life, with a median of 8 days  
• Necrotizing enterocolitis was not associated with low citrulline concentration (p = 0.73)                                                                                                                  |
### Table 2.2  Intestinal transplantation studies.

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<tr>
<th>No</th>
<th>Authors</th>
<th>Settings (sample and design)</th>
<th>Main Results</th>
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</table>
| 1  | Pappas *et al.*  (2001) | - Aim: To investigate impact of acute cellular rejection of intestinal allografts on serum citrulline levels  
- Sample: healthy volunteers (n = 6), patients who underwent small bowel transplant (n = 7)  
- Concurrent measurement of serum citrulline levels with characterization of acute cellular rejection  
- Rejection confirmed by biopsy and graded by standardized criteria | - Controls vs post-transplantation samples: significantly higher mean citrulline concentrations at any rejection grade  
- Mean concentrations declined significantly as rejection severity increased  
- Statistically significant overall downward trend (p < 0.05)  
- In sequential measurements, citrulline levels increased significantly over time with declining severity of rejection  
- Significant increase in mean citrulline concentrations between post-transplant days 3–16 and 52–60 (p < 0.01) |
| 2  | Gondolesi *et al.* (2002) | - Aim: To investigate impact of acute cellular rejection of intestinal allografts on serum citrulline levels  
- Concurrent measurement of plasma citrulline levels with histopathological diagnosis from biopsy (acute cellular rejection (normal, indeterminate, mild, or moderate), viral enteritis (cytomegalovirus or adenovirus), and for other miscellaneous histological diagnoses  
- Sample: 9 consecutive intestinal transplant recipients  
- Thirty-two citrulline measurements | - Mean citrulline levels  
  - overall: 17.5 ± 13.3 μmol/L (range, 0.8 to 68 μmol/L)  
  - normal biopsies: 26 ± 15.7 μmol/L  
  - indeterminate biopsies: 11.9 ± 7.7 μmol/L  
  - mild rejection: 15.4 ± 7.5 μmol/L  
  - moderate rejection: 5.5 ± 0.7 μmol/L  
  - viral enteritis: 9.3 ± 5.85 μmol/L  
  - functional bowel biopsies (n = 22) vs dysfunctional bowel biopsies (n = 10): 19.3 ± 13.6 μmol/L vs 7.8 ± 4.7 μmol/L; p = 0.0001  
- Pearson correlation coefficient between citrulline levels and rejection: −0.425 (p = 0.05)  
- Spearman’s rho correlation coefficient between citrulline levels and rejection: −0.52 (p < 0.01) |
| 3  | Pappas *et al.*  (2002) | - Aim: To investigate impact of rejection of intestinal allografts on serum citrulline levels  
- 10 pre-transplant samples, 11 control specimens, 49 post-transplant samples from 7 patients along with 1 pre-transplant serum sample from each patient and 6 samples from healthy controls, 83 sequential serum samples from 11 patients (5 children, 6 adults), median follow-up 26 days  
- All samples obtained within 3 days of biopsy | - Pre-transplant specimens vs healthy controls: significant difference in mean citrulline (p < 0.01)  
- Mean citrulline levels declined significantly with increasing acute cellular rejection in post-transplant period  
- Mean citrulline levels: pre-transplant: 20.1 ± 10.3 μmol/L vs control: 40.0 ± 7.3 μmol/L (p < 0.01) |
| 4  | Gondolesi *et al.* (2004) | - Aim: To analyse plasma citrulline in intestinal transplant recipients without rejection or other histological abnormalities  
- Sample: 40 patients  
- Plasma citrulline measured with high performance liquid chromatography Beckman amino acid analyser (within 24 h of protocol or clinically indicated endoscopic biopsy procured > 6 and < 360 days post-transplant)  
- Measurements included for analysis corresponded to normal (or minimally abnormal) biopsies that remained so for 7 days  
- Criteria met in: 145 samples, 10 adults and 14 children | - Mean citrulline levels:  
  - overall: 34.0 ± 19.9 μmol/L  
  - between 6 and 30 days post-transplant: 22.2 ± 13.2 μmol/L  
  - between 30 and 60 days post-transplant: 34.9 ± 17.2 μmol/L (p = 0.001)  
  - between 60 and 90 days post-transplant: 43.6 ± 15.8 μmol/L (p = 0.001)  
  - stable until end of first year  
- Plasma citrulline lower in 13 patients with body surface area ≤ 1 m² vs 11 patients with body surface area ≥ 1.1 m² (p = 0.0001)  
- Plasma citrulline increased linearly during first 120 days in both body surface area groups (r = 0.573, r = 0.512; p = 0.0001) |
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<th>No</th>
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<th>Main Results</th>
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<tbody>
<tr>
<td>5</td>
<td>Pappas et al.</td>
<td>• <strong>Aim:</strong> To compare serum citrulline concentrations with biopsy-based grades of rejection</td>
<td>• Median time-to-achieve normal citrulline ($\geq 30 \mu mol/L$): 79 days post-transplant ($n=21$)</td>
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<td></td>
<td>(2004a)</td>
<td>• Sample: 26 isolated intestinal and multivisceral transplant recipients</td>
<td>• Significantly higher maximum grade of rejection after 14 days post-transplant linked to longer time-to-achieve normal citrulline ($p &lt; 0.00001$) and not receiving a multivisceral transplant ($p = 0.0005$)</td>
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<td></td>
<td></td>
<td>• Other factors recorded: patient and donor age and sex, ischaemia time, serum creatinine, type of transplant.</td>
<td>• Normalization of citrulline levels did not occur in some cases with moderate-to-severe rejection</td>
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<td>• Straight-line fitting of citrulline levels over time (stepwise linear regression)</td>
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<td>6</td>
<td>Pappas et al.</td>
<td>• <strong>Aim:</strong> To compare serum citrulline concentrations with biopsy-based grades of rejection</td>
<td>• Time to achieve normal citrulline ($&gt;30 \mu mol/L$): 1-730 days post-transplant ($n = 21$) with increasing citrulline levels over time</td>
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<td></td>
<td>(2004b)</td>
<td>• Sample: 26 isolated intestinal and multivisceral transplant recipients</td>
<td>• Longer time-to-achieve normal citrulline: independent predictor of maximum acute cellular rejection ($p &lt; 0.0001$) and average acute cellular rejection ($p = 0.0005$) 14 days post-transplant.</td>
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<td>• Serum citrulline concentrations determined by ion exchange chromatography and</td>
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<td>compared to biopsy-based grade of acute cellular rejection.</td>
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<tr>
<td></td>
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<td>• Other factors recorded: patient and donor age and sex, ischemia time, serum creatinine, type of transplant.</td>
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<tr>
<td></td>
<td></td>
<td>• Straight-line fitting of citrulline levels over time (stepwise linear regression)</td>
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<tr>
<td>7</td>
<td>Yu et al.</td>
<td>• <strong>Aim:</strong> To investigate correlation between plasma and dried blood spot specimen</td>
<td>• Very strong linear correlation between the plasma and dried blood spot citrulline concentrations ($r = 0.87$, $p &lt; 0.001$)</td>
</tr>
<tr>
<td></td>
<td>(2005)</td>
<td>citrulline concentrations after intestinal transplantation</td>
<td>• Correlation was maintained when evaluating only intestinal transplant recipients</td>
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<td></td>
<td></td>
<td>• Plasma and dried blood spot samples were analysed by hydrophilic interaction</td>
<td>• Sonication time, citrulline concentrations, length of time to assay date: no effect on strength of correlation ($p &gt; 0.05$)</td>
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<td></td>
<td></td>
<td>chromatography tandem mass spectrometry</td>
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<td>• Correlation analysed by type of surgery, sonication time, dried blood spot citrulline</td>
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<td></td>
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<td>levels, the time interval between the blood sample collection and assay date</td>
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<td>8</td>
<td>David et al.</td>
<td>• <strong>Aim:</strong> To determine whether serum citrulline level within 30 days of acute rejection</td>
<td>• Mean serum citrulline levels:</td>
</tr>
<tr>
<td></td>
<td>(2006)</td>
<td>could predict rejection episode</td>
<td>o Mild rejection (12 episodes): 15.0 ± 2.3 μmol/L (prior) vs 18.8 ± 2.4 μmol/L (rejection-free periods) ($p = 0.17$)</td>
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<td></td>
<td></td>
<td>• Comparison of mean citrulline level determined within 30 days of the start of an</td>
<td>o Moderate to severe rejection (25 episodes): 12.4 ± 1.1 μmol/L (prior) vs 18.8 ± 2.0 μmol/L (rejection-free periods) ($p = 0.002$)</td>
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<td></td>
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<td>acute rejection episode against mean citrulline level during a rejection-free period</td>
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<td></td>
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<td>• Sample: 22 patients who experienced 37 episodes</td>
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<td>9</td>
<td>Gondolesi et al.</td>
<td>• <strong>Aim:</strong> To determine sensitivity and specificity of plasma citrulline as diagnostic tool</td>
<td>• Significant mucosal damage vs intestines with no or mild injury: plasma citrulline 22.9 ± 15.1 vs 38 ± 23.2 μmol/L ($p &lt; 0.0001$)</td>
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<tr>
<td></td>
<td>(2006)</td>
<td>for all graft injury</td>
<td>• Sensitivity and specificity of the test were 80% and 58.1% for rejection, and 56.3% and 66% for viral enteritis</td>
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<td>• 403 citrulline samples within 24 h of intestinal biopsy in 49 patients</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• Correlation of citrulline with mucosal damage and histopathological diagnoses</td>
<td></td>
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<tr>
<td>10</td>
<td>David et al.</td>
<td>• <strong>Aim:</strong> to determine citrulline cut-off levels for diagnosis of acute rejection and</td>
<td>• Seven significant predictors of lower citrulline levels were identified: presence of mild, moderate, or severe acute cellular rejection, presence of bacteraemia or respiratory infection; paediatric age; and time from transplant to sample ($p &lt; 0.00001$)</td>
</tr>
<tr>
<td></td>
<td>(2007)</td>
<td>predictors of citrulline levels post-transplant</td>
<td>• A cut-off level citrulline 13 μmol/L had high sensitivity for detecting moderate or severe acute cellular rejection negative predictive value were high (96.4%, 99%; respectively). Specificity was 54% to 74% in children and 83% to 88% in adults.</td>
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<td>• Dried blood spot citrulline samples from 57 intestinal transplant recipients at or</td>
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<td>beyond 3 months post-transplant</td>
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<td>• Stepwise linear regression was performed to determine significant predictors patients’</td>
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<td>citrulline levels</td>
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Table 2.2  (Continued)

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<th>No</th>
<th>Authors</th>
<th>Settings (sample and design)</th>
<th>Main Results</th>
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| 11 | David *et al.* (2008) | - Aim: To determine the significant value of citrulline level in the post-transplant setting, which would correlate with complications of rejection and infection  
- 2,135 dried blood spot citrulline samples were obtained from 57 small intestine transplant recipients three months or more after post-transplant | - A cut-off level citrulline 13 μmol/L had high sensitivity for detecting moderate or severe acute cellular rejection (96.4%)  
- Specificity was high (54%-74% in children and 83%-88% in adults), and the negative predictive value was >99% |
| 12 | Ruiz *et al.* (2010) | - Aim: To evaluate the correlation of plasma citrulline and rejection episodes in intestinal transplantation  
- From January 2007 until present, citrulline was measured from small bowel patients and examined for correlation with rejection status of the graft as defined by graft biopsies  
- 5195 citrulline samples were analysed | - Average serum citrulline levels decreased significantly when patients presented a rejection episode  
  - No rejection: 17.38 μmol/L  
  - Mild rejection, 13.05 μmol/L  
  - Moderate rejection, 7.98 μmol/L  
  - Severe rejection, 6.05 μmol/L |
| 13 | Hibi *et al.* (2012) | - Aim: To investigate the association between citrulline levels acute cellular rejection to identify a cut-off point of citrulline that predicts acute cellular rejection beyond 3 months posttransplant in the paediatric patient population.  
- 13,499 citrulline samples were prospectively collected from 111 consecutive paediatric intestinal/multivisceral transplant recipients. 2,155 were obtained concurrently with intestinal biopsies (1995-2011)  
- 185 acute cellular rejection episodes observed among 74/111 patients (median follow-up: 4.4 years). | - Citrulline levels were inversely proportional to the severity of acute cellular rejection.  
- Negative predictive values for any type of acute cellular rejection (cut-off, 20 μmol/L) and moderate/severe acute cellular rejection (cut-off, 10 μmol/L) were 95% and 99%, respectively.  
- When patients were divided according to graft size, diagnostic accuracy using the same cut-off was identical.  
- Subgroup analysis by the timing of citrulline measurement prior to biopsy varying from 1 to 7 days demonstrated comparable results. |
Table 2.3  Short bowel syndrome studies.  

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<th>Settings (sample and design)</th>
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<tr>
<td>1</td>
<td>Crenn et al. (1998); Crenn et al. (2000)</td>
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</table>
- 57 patients post-absorptive citrulline concentration was measured and parenteral nutrition dependence was used to define permanent (n = 37) and transient (n = 20) intestinal failure  
- Absorptive function, studied over a 3-day period, was evaluated by net digestive absorption for protein and fat  
- Relations between quantitative values were assessed by linear regression analysis and cut-off citrulline threshold, for a diagnosis of intestinal failure by linear discriminant analysis  
|    |                                 |                                            | Short bowel syndrome vs controls (n = 51): 20 ± 13 vs. 40 ± 10 μmol/L (p < 0.001)  
|    |                                 |                                            | Citrulline levels were correlated to small bowel length (p < 0.0001, r = 0.86) and to net digestive absorption of fat, but not to body mass index and creatinine clearance  
|    |                                 |                                            | A cut-off level of 20 μmol/L classified short bowel patients with permanent intestinal failure with high sensitivity (92%), specificity (90%), positive predictive value (95%), and negative value (86%) and was a more reliable indicator (odds ratio 20.0, 95% CI 1.9-206.1) than anatomic variables (odds ratio 2.9, 95% CI 0.5-15.8) to separate transient from permanent intestinal failure  |
| 2  | Pita et al. (2003); Pita et al. (2004) |  
- Sample: 13 short bowel syndrome patients (7 men; 60.2 ± 15.2 years)  
- Groups according to remnant bowel length (Group A: 61-150 cm, n = 6; Group B: > 60 cm, n = 7)  
- Plasma urea-cyclo amino acids, ammonium and urinary orotic acid were determined  
|    |                                 |                                            | Regarding citrulline, Group B levels were significantly lower vs controls (p < 0.001)  
|    |                                 |                                            | Comparisons between patient groups showed higher arginine in Group A (p < 0.05) and non-statistically lower citrulline in Group B  |
| 3  | Kábrt et al. (2003)             |  
- Sample: adult patients with short bowel syndrome (n = 20)  
  o 10 on long-term parenteral nutrition  
  o 10 not on parenteral nutrition  
  o Controls: 9 normal subjects  
- Nutritional assessment with anthropometry and laboratory parameters  
- Post-absorptive plasma concentrations of amino acids determined by ion exchange chromatography  
|    |                                 |                                            | Total amino acids and non-essential amino acids were same in all groups.  
|    |                                 |                                            | Essential amino acid/non-essential amino acid branched-chain amino acid/total amino acid ratios were significantly lower in the short bowel syndrome patient group than in the normal controls.  
|    |                                 |                                            | Concentration of citrulline was significantly lower only in the group of short bowel syndrome patients who had to remain on total parenteral nutrition.  |
| 4  | Gong et al. (2005, 2007)        |  
- Aim: To investigate the significance of serum citrulline in evaluating the remnant small bowel enterocytes mass and absorptive function in short bowel patients  
- Serum citrulline concentrations were determined using high-performance liquid chromatography in 22 short bowel syndrome patients and 33 healthy controls  
- Five-hour urine D-xylose excretion and digestive protein absorption were measured using high-performance liquid chromatography and micro-Kjeldahl method  
|    |                                 |                                            | Serum citrulline levels were significantly lower in short bowel syndrome patients compared with healthy controls  
|    |                                 |                                            | In short bowel syndrome patients, citrulline correlated with remnant small bowel length (r = 0.82, p < 0.001), surface area (r = 0.86, p < 0.001), 5-h urine D-xylose excretion (r = 0.56, p = 0.007), and digestive protein absorption (r = 0.48, p = 0.046).  
|    |                                 |                                            | Citrulline level in six patients receiving rehabilitation therapy correlated with intestinal protein absorption (r = 0.79, p = 0.063) and urine D-xylose excretion (r = 0.81, p = 0.053).  |
| 5  | Rhoads et al. (2005)            |  
- Aim: To determine whether serum citrulline levels correlate with total parenteral nutrition independence in children with short bowel syndrome  
- Study design: serum amino acid profiles over a 24-month interval from all infants with short bowel syndrome 3 weeks to 4 years of age  
- Remaining small intestine length was recorded at surgery, and % of enteral calories tolerated was determined in 24 infants with short bowel syndrome and 21 age-matched controls  
|    |                                 |                                            | In patients with short bowel syndrome, serum citrulline correlated linearly with tolerated enteral calories (r = 0.85, p < 0.001) and bowel length (r = 0.47, p < 0.03)  
|    |                                 |                                            | A citrulline cut-off level of 19 μmol/L had 94% sensitivity and 67% parenteral nutrition independence.  
|    |                                 |                                            | Mean citrulline levels:  
  short bowel syndrome weaned off parenteral nutrition: 30 ± 2 μmol/L  
  short bowel syndrome subsequently weaned off parenteral nutrition: 20 ± 2 μmol/L  
  short bowel syndrome parenteral nutrition: 11 ± 2 μmol/L  
|    |                                 |                                            | Controls: 31 ± 2 μmol/L  |
### Table 2.3 (Continued)

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| 6  | Luo et al. (2007) | • Aim: To examine whether plasma citrulline and glutamine concentrations are biomarkers of residual small intestinal length and nutrient absorptive functions in adult short bowel syndrome patients.  
• Sample: 24 patients on parenteral nutrition in a double-blind, randomized trial of individualized dietary modification ± recombinant human growth hormone.  
• Intestinal absorption studies and plasma measurements of citrulline and glutamine were performed. | • Residual small bowel length was positively correlated with baseline plasma citrulline ($r = 0.467$, $p = 0.028$).  
• No significant correlations between absolute citrulline and glutamine concentrations and the percent absorption of nutrient substrates at any time point were observed.  
• No correlation between the change in citrulline and glutamine concentration and the change in % nutrient absorption was observed. |
| 7  | Nion-Larmurier et al. (2007) | • Twenty-three patients who had a bowel resection and a provisional ileostomy were studied in the month before and months after recovery.  
• Basal citrulline levels were measured before and after restoration of continuity on 16 operated patients and prospectively in 7. | • Citrulline levels (mean ± SD) before recovery were $20.9 ± 8.6 \text{μmol/L (n = 23)}$.  
• Citrulline levels correlated to the length of bowel length ($r = 0.83; p = 0.002$) |
| 8  | Papadia et al. (2006a); Papadia et al. (2006b); Papadia et al. (2007) | • Sample: (a) Crohn’s disease with massive small bowel resection leaving < 50 cm (n = 6), (b) Crohn’s disease with 50-150 cm remaining (n = 9), (c) Crohn’s disease with no resection but active inflammation (n = 7), (d) Crohn’s disease without resection or active inflammation (n = 9), (e) mesenteric infarction with resection leaving < 50 cm (n = 6), (f) mesenteric infarction leaving 50-150 cm (n=6), (g) active coeliac disease (n = 6), (h) healthy volunteers (n = 6).  
• Post-absorptive fasting plasma citrulline was measured using reverse-phase, high performance liquid chromatography. Absorptive capacity and permeability were also measured after oral sugar-mix ingestion. | • Plasma citrulline strongly correlated with small bowel length ($p < 0.0001$) and xylose absorption ($p < 0.001$).  
• No correlation was found with C-reactive protein, permeability, albumin, sedimentation rate, white cell count, or platelet count.  
• Citrulline levels in Crohn’s disease and mesenteric infarction with small bowel length 50-150 cm vs less than 50 cm: $21.0$ vs $9.2 \text{μmol/L (p < 0.0004)}$, respectively. |
| 9  | Peters et al. (2007a, 2007b); Peters et al. (2007c); Peters et al. (2008b) | • Aim: to explore diagnostic value of fasting citrulline concentrations to detect decreased intestinal energy absorption in patients with recently diagnosed coeliac disease (n=15), refractory coeliac disease (n = 9) and short bowel syndrome (n = 16).  
• Fasting plasma citrulline concentrations were determined by high performance liquid chromatography in 40 adult patients and 21 healthy subjects.  
• Intestinal energy absorption capacity using bomb calorimetry was determined. | • Mean citrulline levels:  
  o Refractory celiac disease vs healthy subjects: $28.5 ± 9.9$ vs $38.1 ± 8.0 \text{μmol/L, p < 0.05}$.  
  o Coeliac disease vs healthy subjects $28.5 ± 9.9$ vs $38.1 ± 6.4 \text{μmol/L, p < 0.05}$.  
• Mean intestinal energy absorption capacity:  
  o Short bowel syndrome patients vs healthy subjects: $64.3 ± 18.2$ vs $90.3 ± 3.5\%, p < 0.001$  
  o Refractory celiac disease vs healthy subjects: $64.3 ± 18.2$ vs $82.3 ± 11.7\%, p < 0.01$  
  o Coeliac disease vs healthy subjects $64.3 ± 18.2$ vs $89.2 ± 3.4\%, p < 0.001$.  
• No relation was observed between fasting plasma citrulline concentration and intestinal energy absorption capacity ($r=0.09, P=0.56$, area under the ROC curve $0.50$). |
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| 10 | Parekh et al. (2008) | • Sample: 49 healthy controls with an intact gastrointestinal tract and no known metabolic or digestive diseases and 30 short bowel syndrome (< 200cm small bowel) patients dependent on parenteral nutrition.  
• Venous post-absorptive plasma amino acid concentrations were measured in all subjects after an 8 hour fast. | • Mean citrulline levels: Short bowel syndrome patients vs healthy controls: 21.4 vs 33.2 μmol/L, p = 0.0002.  
• Area under the ROC curve was 0.82 (95% CI 0.71, 0.93) and a citrulline cut-off of 20 μmol/L had 100% specificity and 56.6% sensitivity.  
• Citrulline increased by 1.65 μmol/L with every 5 year increase in age (p = 0.044).  
• Citrulline increased by 4.9 μmol/L for every 50cm increase in small bowel length (p = 0.018).  
• Citrulline decreased by 9 μmol/L for every 1000 kcal/day increase in parenteral nutrition (p < 0.0001). |
| 13 | Santarpia et al. (2008) | • Sample: 25 patients with short bowel syndrome after at least 18 months since last digestive circuit modification; 24 of them were again evaluated 1 year later.  
• Ten patients were weaned off parenteral nutrition and 15 were dependent on parenteral nutrition.  
• Fifty-four healthy volunteers (28 women and 26 men) served as controls. | • Five amino acids (citrulline, leucine, isoleucine, valine and tyrosine) were significantly lower in all short bowel syndrome patients versus controls, whereas glutamine was significantly higher.  
• Only serum citrulline measured was significantly related to small bowel length. |
| 14 | Bailly-Botuha et al. (2009) | • Prospective plasma citrulline assays were performed in 31 children with short bowel syndrome.  
• Median age was 16 months and median follow-up was 14 months. | • Plasma citrulline at inclusion showed a positive correlation with residual short bowel length.  
• Follow-up values correlated negatively with intestinal failure severity.  
• Plasma citrulline increased over time during or after weaning from parenteral nutrition (from 15.8 ± 11.5 μmol/L to 19.3 ± 3.8 μmol/L) but remained stable and low in patients who continued on parenteral nutrition (6.5 ± 3.0 μmol/L at inclusion and 7.7 ± 6.0 μmol/L at last follow-up). |
| 15 | Fitzgibbons et al. (2009) | • Aim: To evaluate the relationship between citrulline and parenteral nutrition independence in children with short bowel syndrome.  
• Sample: Retrospective review of all patients in a multidisciplinary paediatric intestinal rehabilitation clinic with a recorded citrulline between January 2005 and December 2007 (n = 27). | • Citrulline levels correlated positively with bowel length (r = 0.73; p < 0.0001) and were a strong predictor of parenteral nutrition independence (p = 0.002; area under the ROC curve = 0.88; 95% CI 0.75-1.00).  
• Optimal citrulline cut-off point distinguishing patients who reached parenteral nutrition independence was 15 μmol/L (sensitivity = 89%; specificity = 78%). |
| 16 | Gong et al. (2009) | • Aim: To evaluate long-term clinical significance of enteral nutrition in weaning adult short bowel patients off parenteral nutrition undergoing intestinal rehabilitation therapy.  
• Sample: 61 adult patients with small bowel length 47.95 ± 19.37 cm were retrospectively analysed. | • Nutritional and anthropometric parameters, urine 3-hr D-xylene excretion and serum citrulline levels all increased significantly after intestinal rehabilitation therapy and on follow-up compared with baseline. |
| 17 | Picot et al. (2010) | • Twenty-six patients with small bowel disruption and double enterostomy were treated with chyme reperfusion.  
• Faecal wet weight, nitrogen and fat absorption, parenteral nutrition delivery and citrulline were measured before and after the initiation of chyme reperfusion with a median follow-up of 30 days. | • Chyme reperfusion decreased the intestinal wet weight output and parenteral nutrition dependence.  
• Chyme reperfusion was associated with a rise in net nitrogen and fat digestive absorption and citrulline (17.0 ± 10.0 vs 31.0 ± 12.0 μmol/L, p = 0.0001).  
• Before the initiation of chyme reperfusion, citrulline levels correlated positively with the absorptive post-duodenal small bowel length (r = 0.39, p = 0.04), but not with the total post-duodenal small bowel length (r = 0.11, P = 0.60). |
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| 18 | Noto et al. 2008a; Noto et al. 2008b; Diamanti et al. 2010; Diamanti et al. 2011b | • Sample: Between March 2005 and March 2010, 28 short bowel syndrome patients on parenteral nutrition  
• Citrulline levels and enteral intake determinations were measured on inclusion and at 6-month intervals | • Citrulline significantly correlated with the residual duodenum-jejenum length ($r^2 = 0.22$, $p = 0.0113$) and with enteral intake ($r^2 = 0.20$, $p = 0.016$, $r^2 = 0.48$, $p = 0.0001$)  
• Baseline citrulline over 10 μmol/L and a longitudinal increase >25% provided a weak association with bowel adaptation (likelihood ratios 2.6 and 2.4, respectively), unlike residual small bowel length ≥ 20 cm and the presence of > 50% of the colon. |
| 19 | Khan et al. 2011             | • Sample: Serum citrulline was measured in 19 subjects with short bowel syndrome; 10 females and 17 were on parenteral nutrition  
• Age: 7 months to 21 years; Bowel length: 5 to 150 cm, and percentage of parenteral nutrition providing 0-100% of caloric intake. | • Citrulline levels decreased with increased parenteral nutrition intake ($r = 0.69$)  
• Citrulline levels correlated with bowel length ($r = 0.73$) |
| 20 | Pironi et al. 2005; Pironi et al. 2011; Pironi et al. 2012 | • Sample: Nineteen healthy subjects and 93 short bowel syndrome patients were studied, 67 on home parenteral nutrition and 26 stable on oral diet | • Mean citrulline levels:  
• Healthy subjects: 37 μmol/L  
• Short bowel syndrome patients on oral diet: 33 μmol/L  
• Short bowel syndrome patients on home parenteral nutrition 20 μmol/L ($p < 0.001$).  
• Citrulline cut-off of 14 μmol/L had sensitivity 49%, specificity 100%, $p < 0.001$; for distinguishing between short bowel syndrome on parenteral nutrition vs oral diet |
| 21 | Raphael et al. 2011           | • Design: Open-labelled pilot study in a limited access program for cisapride.  
• Indications were short bowel syndrome with underlying dysmotility and difficulty advancing enteral feeds despite standard therapies and without evidence of anatomic obstruction.  
• Patients received cisapride 0.1 to 0.2 mg/kg per dose for 3 to 4 doses per day. | • Ten patients were enrolled in a multidisciplinary paediatric intestinal rehabilitation program.  
• Median (IQR) residual bowel length was 102 (85-130) cm. Median (IQR) citrulline level was 14.5 (10.5-31.3) μmol/L.  
• Seven patients improved in enteral tolerance during treatment and 2 were weaned completely from parenteral nutrition. |
| 22 | Suzuki et al. 2012           | • Design: To measure plasma citrulline in six patients with intestinal dysfunction who were in the acute and chronic phase for more than 6 months. | • Four patients out of six could be withdrawn from total parenteral nutrition, and their plasma citrulline level increased up to 15 μmol/L.  
• Two patients, who could not be withdrawn from parenteral nutrition, showed very low levels of plasma citrulline throughout the treatment course (under 15 μmol/L) |
| 23 | Amiot et al. 2013           | • Sample: 268 non-malignant short bowel syndrome patients  
• Home parenteral nutrition dependence and survival rate were studied with univariate and multivariate analysis. | • Home parenteral nutrition dependence was significantly decreased with an early (<6 months) plasma citrulline concentration >20 μmol/L, a remaining colon >57% and a remnant small bowel length >75 cm |
| 24 | Pinto Costa et al. 2013     | • Sample: Case-control study, 11 patients with short bowel syndrome, 13 patients submitted to malabsorptive bariatric surgery and 11 healthy controls.  
• Plasma levels of amino acids were determined, before and after a stimulation test with oral L-glutamine, by ion exchange chromatography. | • Citrulline levels were lower in short bowel patients ($28.6 ± 11.3$ vs $35.5 ± 11$ in operated obese vs $32.2 ± 6.6$ μmol/L in controls; $p > 0.05$) and lower than $25.5$ μmol/L in $54.5%$ of them  
• Relative variation of citrulline levels at the 80th minute of test was lower in short bowel patients with high predictive capacity of a short bowel ≤ 50 cm (area under ROC curve = 0.825; $p = 0.038$). |
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| 25 | Vecino Lopez et al. (2013) | - Plasma citrulline concentration was determined by chromatography in 57 patients (age 0.5-18 years) admitted to the Intestinal Rehabilitation Unit with intestinal failure.  
  - Group I: short bowel syndrome totally dependent on parenteral nutrition  
  - Group II: short bowel syndrome under mixed enteral-parenteral nutrition  
  - Group III: Intestinal failure weaned off parenteral nutrition after a rehabilitation period  
  - Group IV: small bowel transplanted patients weaned off parenteral nutrition and on a normal diet | - Mean plasma citrulline levels:  
  - Group I (n = 15): 7.1 ± 4.1 μmol/L  
  - Group II (n = 11): 15.8 ± 8.9 μmol/L  
  - Group III (n = 13): 20.6 ± 7.5 μmol/L  
  - Group IV (n = 25): 28.8 ± 10.1 μmol/L  
  - Values were significantly lower in group I compared to groups II-IV (p < 0.001), and in group II compared to groups III-IV (p < 0.001). Citrulline was correlated with remnant small bowel length (r = 0.85, p < 0.05).  
  - In group IV citrulline levels decreased >50% in 3 patients who developed moderate-severe rejection, and in one patient who developed viral enteritis |
| 1  | Buchman et al. (2010) | - Design: Subjects with moderate-to-severe Crohn’s disease randomized to placebo or 1 of 3 doses of teduglutide (0.05, 0.10, or 0.20 mg/kg daily) (n = 100).  
  - Primary outcome: the percentage of subjects in each group that responded to treatment, defined as a decrease in Crohn’s Disease Activity Index score | - Mean baseline Crohn’s Disease Activity Index score was 290.8 ± 57.6, similar across groups.  
  - Plasma citrulline was similar across groups at baseline, but increased substantially over time in all teduglutide groups when compared with placebo at week 8 |
| 2  | Jeppesen et al. (2011); Seidner et al. (2015) | - Sample: 83 patients randomised to receive subcutaneous teduglutide 0.10 mg/kg/day (n = 32), 0.05 mg/kg/day (n = 35) or placebo (n = 16) once daily.  
  - Responders were subjects who demonstrated reductions of ≥ 20% in parenteral volumes from baseline at weeks 20 and 24 | - Three teduglutide-treated patients were completely weaned off parenteral support.  
  - Villus height, plasma citrulline concentration and lean body mass were significantly increased with teduglutide compared with placebo |
| 3  | Gilroy et al. (2009); Jeppesen et al. (2009); Jeppesen et al. (2012); Seidner et al. (2015) | - Design: 24-week study of short bowel syndrome patients who were given subcutaneous teduglutide (0.05 mg/kg/d, n = 43) or placebo (n = 43) once daily.  
  - Parenteral support was reduced if 48-hour urine volumes exceeded baseline values by ≥ 10%.  
  - The primary efficacy end point was number of responders | - There were significantly more responders in the teduglutide group (27/43) than the placebo group (13/43, p = 0.002).  
  - At week 24, the mean reduction in parenteral support volume in the teduglutide group was 4.4 ± 3.8 L/week compared with 2.3 ± 2.7 L/week in the placebo group (p < 0.001).  
  - Teduglutide increased plasma concentrations of citrulline, a biomarker of mucosal mass. |
| 4  | Naimi et al. (2013) | - Sample: Eight short bowel syndrome patients (5 Females, 60 ± 7 years; remnant small bowel 111 ± 62 cm)  
  - Design: open-label, sequential study comparing continuous GLP-2 vs three times per day GLP-2  
  - Post-absorptive plasma citrulline, reflecting enterocyte mass, was measured by high performance liquid chromatography. | - Both GLP-2 dosing regimens reduced diarrhoea and increased wet weight absorption  
  - Significant increases in plasma citrulline (continuous GLP-2: 7.5 ± 7 μmol/L and three times per day GLP-2: 12.7 ± 8 μmol/L, p = 0.001) suggesting intestinotrophic effects in relation to GLP-2 treatment, are followed by increases in relative absorption of energy, carbohydrate and fat. |
### Table 2.4  Coeliac disease, Crohn’s disease and enteropathy studies.

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| 1  | Crenn et al. (2003) | • Aim: To evaluate citrulline as a marker of severity and extent of villous atrophy in patients without intestinal resection.  
  • Sample:  
    - 42 patients with coeliac disease and 10 patients with non-celiac villous atrophy disease were studied by plasma postabsorptive citrulline and biological dosages, biopsies of proximal (duodenojejunal) small bowel and distal ileum (n = 25), or measurement of vitamin B12 absorption (n = 4).  
    - 9 patients were re-evaluated after following a gluten-free diet for 1 year  
    - Controls: 51 healthy subjects and 10 severely malnourished patients with anorexia nervosa with no intestinal mucosal abnormalities | • Plasma citrulline concentrations: Villous atrophy vs healthy subjects: 24 vs 40μmol/L, p < 0.001  
  • Three cut-offs identifies:<10 μmol/L for patients with diffuse total villous atrophy, 10-20 μmol/L for patients with proximal-only total villous atrophy, and 20-30 μmol/L for patients with partial villous atrophy  
  • Plasma citrulline concentration was correlated to the severity and extent of villous atrophy (r = 0.81; p < 0.001) and to albumin levels (r = 0.47; p < 0.01).  
  • Receiver operating characteristic curves indicated that plasma citrulline concentration was the best biological variable to predict villous atrophy  
  • Following a 1-year gluten-free diet, plasma citrulline concentration increased in histologically responsive but not in unresponsive patients |
| 2  | Hozyasz et al. (2006) | • Aim: To determine amino acid concentrations in coeliac disease patients on gluten-free diet and gluten-containing diet  
  • Sample: 61 patients with coeliac disease  
  • Whole blood citrulline were determined in dried blood spots by tandem mass spectrometry | • Mean citrulline levels were higher in patients on strict gluten-free diet comparing to those newly diagnosed (32.2 ± 8.7 vs 24.9 ± 5.7 μmol/L; p=0.025) |
| 3  | Papadia et al. (2006a); Papadia et al. (2006b); Papadia et al. (2007) | • See Table 2.3 for details | |
| 4  | Peters et al. (2007a, 2007b); Peters et al. (2007c); Peters et al. (2008b) | • See Table 2.3 for details | |
| 5  | Miceli et al. (2008) | • Sample: 50 healthy volunteers, 21 patients with untreated coeliac disease and 6 patients with refractory coeliac disease  
  • Serum citrulline levels and duodenal lesions were evaluated at the time of diagnosis, and after at least 24 months of gluten-free diet  
  • Serum citrulline concentrations were determined by ion exchange chromatography. | • In comparison to healthy volunteers, serum citrulline concentrations were significantly lower in untreated and refractory coeliac disease patients  
  • No significant difference was found between untreated and refractory coeliac disease patients and between patients with different patterns of clinical presentation or various degrees of duodenal lesions  
  • After a gluten-free diet, mean serum citrulline concentration increased |
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| 6  | Crenn et al. (2009a); Crenn et al. (2009b) | • Sample: 6 groups of HIV patients (n = 115): 1) undetectable viral load without chronic diarrhoea (a; n = 40) and with protease inhibitor-associated toxic chronic diarrhoea (b; n = 26), 2) detectable viral load and CD4 > 200/mm³ without (a; n = 6) and with (b; n = 11) chronic diarrhoea, and 3) detectable viral load and CD4 <200/mm³ without chronic diarrhoea (a; n = 7) and with opportunistic intestinal infections or HIV enteropathy (b; n = 25)  
• The influence of diarrhoea on citrulline was assessed by comparing subgroups a and b with healthy control subjects (n = 100). | • Citrulline was slightly decreased (22-30 μmol/L) in groups 1b and 2b  
• Citrulline levels in control subjects vs patients without chronic diarrhoea (groups 1a, 2a, and 3a): 38 ± 8 vs 36 ± 6 μmol/L.  
• In group 3b, a citrulline concentration <10 μmol/L was associated with a clinical indication for parenteral nutrition (p < 0.05).  
• Citrulline correlated positively with albumin (p < 0.01) and BMI (p < 0.05) and negatively with C-reactive protein (p < 0.01).  
• When anti-infectious and nutritional therapies were successful, citrulline normalized in 2-12 weeks |
| 7  | Papadia et al. (2009a); Papadia et al. (2009b); Papadia et al. (2010) | • Post-absorptive fasting serum citrulline was measured in 150 tropical enteropathy patients (n = 44, HIV) with reverse phase, high performance liquid chromatography.  
• Absorptive capacity and permeability were measured after intrajejunal instillation of 4 sugars with assay by thin-layer chromatography.  
• Morphometric analysis was carried out on jejunal biopsies | • HIV positive vs HIV negative patients: median serum citrulline 19 (17-24) μmol/L; p < 0.001  
• There were statistically significant correlations (p < 0.005) between citrulline and: crypt depth; villous height/crypt depth ratio; Shenk-Klipstein score; and xylose absorption, only in the HIV positive |
| 8  | Panetta et al. (2010); Diamanti et al. (2011a) | • Sample: 31 Crohn’s disease patients and 44 controls (2008-2010)  
• Analysis: Differences between groups, at baseline, in plasma citrulline and glutamine and between their baseline and final values during the prospective survey, and correlation between baseline values of citrulline and duration of disease, C-reactive protein, and faecal calprotectin | • Mean citrulline value  
• Controls vs Crohn’s disease: 33.0 ± 7.5 vs 23.5 ± 8.4 μmol/L (p < 0.0001)  
• Crohn’s disease patients with small bowel disease vs ileo-colonic disease: 14.2 ± 5.5 vs 24.7 ± 8.0 μmol/L, p = 0.0037  
• Citrulline ≤ 22 μmol/L had sensitivity of 100% and specificity of 98% for differentiating control subjects from Crohn’s disease patients with small bowel disease |
| 9  | Bernini et al. (2011) | • Sample: 61 overt coeliac disease patients, 29 patients with potential coeliac disease, and 51 control subjects were examined by proton nuclear magnetic resonance of their serum and urine | • Potential coeliac disease largely shares the metabonomic signature of overt coeliac disease. Most metabolites found to be significantly different between control and coeliac disease subjects were also altered in potential coeliac disease |
| 10 | Blasco Alonso et al. (2011) | • Sample and design: Observational case-control study longitudinal in children 16 months to 14 years: 48 with untreated coeliac disease, 9 coeliac children under gluten free diet and 35 non-coeliac healthy children.  
• Plasma amino acids concentration was measured along with other clinical and analytical data | • Cases vs Controls: citrulline, arginine and glutamine 17.7 ± μmol/L, 38.7 ± μmol/L, 479.6 ± μmol/L respectively vs 28.9 ± μmol/L, 56.2 ± μmol/L, 563.7 ± μmol/L.  
• Citrulline levels are significantly lower in the severe degrees of atrophy vs mild ones (13.8 μmol/L vs 19.7 μmol/L, p < 0.05) |
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<td>15</td>
<td>Sevinc <em>et al.</em></td>
<td>• Sample: 62 children with classic coeliac disease, 62 age and sex matched healthy control</td>
<td>• Coeliac children had significant lower plasma levels of citrulline, glutamine and cystine than controls <em>(p &lt; 0.05)</em></td>
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<td></td>
<td>(2015)</td>
<td>• Plasma amino acid levels measured with tandem mass spectrometry.</td>
<td>• Alanine, asparagine, glutamic acid, hydroxyproline, isoleucine, leucine, phenylalanine, proline, serine, threonine and valine were significantly higher in coeliac children than in controls <em>(p &lt; 0.05)</em></td>
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<td>• No significant difference in levels of arginine, argininosuccinate, aspartic acid, glycine, homocysteine, hydroxylysine, lysine, methionine, ornithine, tryptophan, tyrosine, histidine levels between celiac children and healthy controls <em>(p &gt; 0.05)</em></td>
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Table 2.5  Gastrointestinal toxicity from chemo-radiation therapy studies.

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<th>Authors</th>
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<th>Main Results</th>
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</table>
| 1  | Lutgens et al. (2002); Blijlevens et al. (2004) | Sample: 32 haematopoietic stem cell transplant recipients following intensive myeloablative therapy during the first 3 weeks after transplantation when patients have oral mucositis | Significant decline in serum concentrations of citrulline following intensive myeloablative therapy during the first 3 weeks after transplantation when patients have oral mucositis and a markedly disturbed gut integrity  
  Closer inspection of citrulline concentrations of 12 patients confirmed that decline corresponded to the onset of oral mucositis and altered gut integrity |
| 2  | Lutgens et al. (2004)    | Sample: 23 patients were studied weekly during treatment and at intervals of 2 weeks and 3 and 6 months after treatment by post-absorbptive plasma citrulline concentration and clinical toxicity grading.  
  The interrelationship between these variables and the correlation with small-bowel dose and volume parameters were investigated. | During fractionated radiotherapy, citrulline concentration significantly decreased as a function of the radiation dose (p < 0.001) and the volume of small bowel treated (p = 0.001)  
  Plasma citrulline concentration correlated with clinical toxicity during the last 3 weeks of treatment. As a whole, citrulline concentration correlated better with radiation dose and volume parameters than clinical toxicity grading. |
| 3  | Blijlevens et al. (2005a) | Sample: 32 haematopoietic stem cell transplant recipients following intensive myeloablative therapy. | Significant increase of interleukin-8, lipopolysaccharide-binding protein and C-reactive protein indicating mucosal barrier injury as measured by gut integrity, daily mucositis score and serum citrulline concentrations |
| 4  | Blijlevens et al. (2005b) | Design: Prospective, randomised, double-blinded, placebo-controlled pilot study of parenteral nutrition supplemented with 0.57 g/kg glutamine-dipeptide in a homogeneous group of 32 allogeneic stem cell transplant recipients to determine its effect on mucosal barrier injury  
  Mucosal barrier injury measured by sugar permeability tests, daily mucositis score, daily gut score, and citrulline concentrations | The daily gut score was significantly lower for the glutamine group on day 7 post-transplant (p = 0.001) whilst citrulline was lower (p = 0.03) for the placebo group on day 21 post-transplant  
  Albumin was significantly lower in the placebo group on day 21 post-transplant (32 ± 4 vs 37 ± 3, p = 0.001) whilst C-reactive protein was higher (74 ± 48 vs 34 ± 38, p = 0.003) |
| 5  | Lutgens et al. (2005)    | Design: Prospective study, 10 patients with haematological malignancies who were receiving myeloablative therapy had gut toxicity assessed with sugar permeability tests.  
  Serum citrulline concentrations also were determined using archival serum samples | Sensitivity and specificity were better for the citrulline assay compared with sugar permeability tests  
  Maximum gut damage assessed with the citrulline assay was observed 1-2 weeks earlier compared with the sugar permeability test  
  Citrulline indicated recovery of gut damage at 3 weeks after transplantation, whereas most sugar permeability tests remained abnormal |
| 6  | Herbers et al. (2008)    | 29 patients with high-dose melphalan 200 mg/m² to prepare for an autologous Peripheral blood stem cell transplantation  
  Plasma samples from each patient starting before the myeloablative regimen and three times per week thereafter until discharge | Baseline citrulline concentration was 27.6 ± 4.0 μmol/L, and citrulline concentrations declined rapidly thereafter reaching a nadir averaging 6.7 ± 2.7 μmol/L, 12 days after starting melphalan.  
  Citrulline concentrations, only increased gradually and were still low (12 ± 4 μmol/L) at discharge.  
  Their mean citrulline concentrations were lower at 5.5 ± 1.5 μmol/L than were those of patients without bacteremia (10.2 ± 3.9 μmol/L) |
| 7  | Wedlake et al. (2008)    | Sample: 59 patients (30 males) with mixed pelvic malignancies, receiving 45-70 Gy were recruited  
  At baseline and weeks 4 or 5 of radiotherapy, blood samples for citrulline, C-reactive protein, eosinophil cationic protein and stool samples for faecal calprotectin were obtained | Citrulline (p = 0.02) and faecal calprotectin (p = 0.01) values changed significantly between baseline and 4 or 5 weeks.  
  Inflammatory Bowel Disease Questionnaire - Bowel Subset fell significantly (mean fall = 10 points). Changes in markers did not correlate with symptoms. |
### Table 2.5  (Continued)

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| 8  | Derixx et al. (2009)     | • Sample: 34 adult patients with haematological malignancy received allogeneic haematopoietic stem cell transplant 12 days after myeloablative conditioning with a regimen known to induce oral and intestinal mucosal barrier injury  
  • Serum levels of citrulline, intestinal fatty acid binding protein and ileal bile acid-binding protein were measured on transplant days -12, -6, 0, +7, +14 and +21. | • Myeloablative conditioning resulted in a significant decrease in serum citrulline with the nadir on day 7 post-transplant; thereafter, levels rose gradually.  
  • A significant decrease in intestinal fatty acid binding protein and ileal bile acid-binding protein levels occurred from the day of transplant until day +14. |
| 9  | van Vliet et al. (2009)  | • Sample: Children with acute myeloid leukaemia  
  • Investigations: various mucosal barrier injury-related clinical and laboratory tests, reflecting clinical severity (NCI symptomatic adverse events criteria), daily gut score, inflammation (plasma and faecal interleukin-8, faecal calprotectin), enterocytic loss (plasma citrulline, ratio faecal human DNA/total DNA) and intestinal permeability (sugar absorption tests) | • Intestinal mucosal barrier injury as detected by the NCI adverse events criteria was found in 55% of chemotherapy cycles, correlating well with the continuous daily gut score (n = 55, r = 0.581; p < 0.001)  
  • Intestinal cell loss as measured by the ratio faecal human DNA/total DNA and plasma citrulline correlated well with both NCI criteria (r = 0.357, p = 0.005; r = -0.482, p < 0.001) and daily gut score (r = 0.352, p = 0.009; r = -0.625, p < 0.001)  
  • Plasma interleukin-8 correlated strongly to plasma citrulline (r = -0.627; p < 0.001). |
| 10 | Herbers et al. (2010)    | • Sample and design: Citrulline concentrations were determined at baseline and at least once weekly after the start of myeloablative chemotherapy until 30 days thereafter among 94 allogeneic or autologous haematopoietic stem-cell transplant recipients.  
  • Intestinal mucosal damage was described either by level of citrulline on each day, on the basis of different thresholds of citrulline indicating the severity of villous atrophy, or by area under the curve using reciprocal value of 10/citrulline. | • Regimens that incorporated idarubicin induced the most severe intestinal toxicity.  
  • Scores based on the level of citrulline, using severity thresholds, and on the area under the reciprocal curve are able to discriminate between the damage induced by different high-dose chemotherapy regimens. |
| 11 | Jakobsson et al. (2010)  | • Sample: 29 women undergoing pelvic radiotherapy for anal or uterine cancer were prospectively followed  
  • Fatigue and diarrhoea were assessed using patient self-reported questionnaires  
  • Plasma citrulline concentration, as a sign of intestinal injury, and C-reactive protein, orosomucoid, albumin, alpha-1-antitrypsin, and haptoglobin, as signs of systemic inflammation, were analysed. | • Fatigue increased significantly (p < 0.001) and citrulline decreased significantly (p < 0.001) during treatment.  
  • A significant negative correlation (r = -0.40; p < 0.05) was found between fatigue and epithelial atrophy in the intestine (as assessed by plasma citrulline) after 3 weeks of treatment and a significant positive correlation (r = 0.75; p < 0.001) was found between fatigue and diarrhoea. |
| 12 | van der Velden et al. (2010); van der Velden et al. (2013) | • Sample: Retrospective analysis in 163 stem-cell transplant recipients of which data had been collected prospectively on intestinal damage (citrulline), inflammation (C-reactive protein), and neutrophil count.  
  • Six different conditioning regimens were studied; 5 myeloablative and 1 non-myeloablative  
  • Linear mixed model multivariate and AUC analyses were used to define the role of intestinal damage in post-SCT inflammation. | • In the 5 myeloablative regimen there was a striking pattern of inflammatory response that coincided with the occurrence of severe intestinal damage  
  • This contrasted with a modest inflammatory response seen in the non-myeloablative regimen in which intestinal damage was limited.  
  • With linear mixed model analysis the degree of intestinal damage was shown the most important determinant of the inflammatory response, and both neutropenia and bacteraemia had only a minor impact.  
  • AUC analysis revealed a strong correlation between citrulline and C-reactive protein (r = 0.96).  
  Intestinal damage was associated with the occurrence of bacteraemia and acute lung injury, and influenced the kinetics of acute graft-versus-host disease. |
Table 2.5  (Continued)

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<td>13</td>
<td>Onal et al. (2011)</td>
<td>Sample: 53 patients (36 prostate cancer, 17 endometrial cancer) who received 45 Gy pelvic radiotherapy using conventional fractionation. Patients with prostate cancer received an additional 25-30.6 Gy conformal boost. Plasma citrulline levels were assessed on day 0, mid- (week 3) and post-radiotherapy (week 8), and four months post-radiotherapy. Dose-volume histogram, citrulline concentration changes, and weekly intestinal toxicity scores were analysed.</td>
<td>Citrulline concentrations were significantly reduced at week 3 (22.4 ± 5.9 μmol/L; p &lt; 0.0001), treatment end (29.9 ± 8.8 μmol/L; p &lt; 0.0001), and four months post-treatment (34.3 ± 12.1; p = 0.01). The following factor pairs were significantly positively correlated: Citrulline concentration/mean bowel dose during, end of treatment, and four months post-radiotherapy; dose-volume parameters/citrulline change groups; cumulative mean radiation dose/intestinal toxicity at end and four months post-radiotherapy; citrulline changes/intestinal toxicity during and end of radiotherapy. Citrulline concentration changes significantly differed during treatment according to radiotherapy oncology group intestinal toxicity grades (p &lt; 0.0001).</td>
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<td>14</td>
<td>Vokurka et al. (2013)</td>
<td>Sample: prospective study in 11 adults (18 blood samples) with diarrhoea developed after allogeneic stem-cell transplant in between 2011-2012 compared to 20 healthy control samples.</td>
<td>Transplanted patients vs healthy controls: median (IQR) 9.3 (3.62-15.38) vs. 33.3 (26.82-36.23) μmol/L, p&lt;0.0001. Post-transplant toxic intestinal mucositis (n=8, days 1-22 post-transplant) vs. intestinal graft versus host disease (n=7, day 43-142) vs. other aetiology of diarrhoea (n=3, day 120-127): 9.55 (2.95-12.03) vs. 5 (3.85-9.05) vs. 15.6 (15.45-18.3) μmol/L, (p &lt; 0.05).</td>
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<td>15</td>
<td>Gosselin et al. (2014)</td>
<td>Sample: Multicentre, prospective cohort study of 26 children to define time-related changes in serum citrulline during the course of hematopoietic cell transplantation. Markers of gastrointestinal function including oral energy intake, emesis, stool volume, presence of graft-versus-host disease, oral mucositis severity, and cytokine and neurohormone levels were measured. Weekly serum citrulline concentrations were obtained from 10 days prior until 30 days after hematopoietic cell transplantation.</td>
<td>Mean baseline citrulline concentration was 22.7 μmol/L (95% CI 17.7-27.6) on day -10, which decreased to a nadir of 7.5 μmol/L (95% CI 3.1-18.0, p = 0.017) on day 8 following hematopoietic cell transplantation before returning to baseline by day 30. After adjustment for interleukin-6 level (1.0% lower citrulline per 10% increase in interleukin-6, p = 0.004), presence of acute graft-versus-host disease (27% lower citrulline, p = 0.025), and oral energy intake (2.1% lower citrulline per 10% decrease in energy intake, p = 0.018), the nadir shifted to day 10, when mean citrulline concentration was lower in patients with severe oral mucositis (6.7 μmol/L, 95% CI 3.4-13.1) than in those without severe mucositis (11.9 μmol/L, 95% CI 5.8-24.4, p = 0.003). Change in citrulline was not correlated with stool volume, C-reactive protein, tumour necrosis factor-alpha, lepton, or ghrelin.</td>
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<td>16</td>
<td>Karlik et al. (2014)</td>
<td>Aim: To determine whether citrulline levels correlate with clinical markers of intestinal injury in children undergoing a myeloablative allogeneic transplant regimen.</td>
<td>For every 1 μmol/L increase in citrulline, the odds of developing mucositis were 0.88 (95% CI 0.79-0.99, p = 0.036). The odds of developing diarrhoea were 0.70 times less for every 1 μmol/L increase in citrulline (95% CI=0.59-0.84, p &lt; 0.0001).</td>
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<td>17</td>
<td>Brady et al. (2015)</td>
<td>Sample: 15 patients treated with external beam radiation therapy to either prostate only (n=6) or prostate and pelvis (n=9). Plasma citrulline levels were measured prior to radiotherapy and weekly during treatment and at 6 weeks, 3 months and 6 months post external beam radiation therapy. Bowel toxicity was assessed at the same time points using EPIC bowel summary scores.</td>
<td>The strongest correlation between the fall in plasma citrulline levels from baseline and greatest bowel toxicity was observed after 3 weeks of radiotherapy (p=0.03). A strong predictive trend was noted with positive correlations at 6 weeks post radiotherapy (r = 0.594, p = 0.025), 3 months post radiotherapy (r = 0.334, p = 0.060), 6 months post radiotherapy (r = 0.606, p = 0.037), 9 months post radiotherapy (r = 0.618, p = 0.019) and 1 year post radiotherapy (r =0.358, p = 0.345). No significant correlation was found between changes in plasma citrulline levels or EPIC reported toxicity.</td>
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<td>18</td>
<td>Kong et al.</td>
<td>- Sample: 42 patients with gastric or colorectal cancer underwent chemotherapy</td>
<td>- The urinary lactulose-mannitol ratio and plasma citrulline levels increased on the third and sixth post-chemotherapy days, respectively</td>
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<td>(2015)</td>
<td>- Patients were asked to grade and record their symptoms of gastrointestinal toxicity daily</td>
<td>- There were no significant differences in the plasma levels of D-lactic acid, endotoxin or diamine oxidase activity compared to their levels before chemotherapy</td>
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<td>- The urinary lactulose-mannitol ratio was measured to assess the intestinal permeability.</td>
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<td>- Plasma levels of citrulline, diamine oxidase, D-lactic acid, and endotoxin were also measured</td>
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<td>19</td>
<td>Wang et al.</td>
<td>- Aim: To investigate the correlations between fatigue, diarrhoea, and alterations in gut microbiota induced by pelvic radiotherapy.</td>
<td>- During the 5-week treatment of pelvic radiotherapy in 11 cancer patients, the general fatigue score significantly increased and was more prominent in the patients with diarrhoea.</td>
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<td>(2015)</td>
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<td>- The fatigue score was closely correlated with the decrease of serum citrulline and the increases of systemic inflammatory proteins, including haptoglobin, orosomucoid, alpha-1-antitrypsin and tumour necrosis factor-alpha.</td>
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<td>20</td>
<td>Zezulová et al.</td>
<td>- Design: Plasma citrulline, serum neopterin and urinary neopterin were measured weekly in 49 patients with rectal carcinoma during chemoradiation</td>
<td>- Citrulline significantly (p &lt; 0.05) decreased while serum and urinary neopterin concentrations increased during therapy.</td>
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<td>(2016)</td>
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<td>- Irradiated gut volume correlated significantly inversely with citrulline and positively with urinary neopterin.</td>
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<td>- Statistically significant inverse correlations were also observed between urinary neopterin and plasma citrulline concentrations during the treatment.</td>
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<td>- Urinary neopterin concentrations were significantly higher and citrulline concentrations were lower in patients who experienced grade ≥ 3 gastrointestinal toxicity</td>
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### Table 2.6: Studies regarding citrulline levels in critical illness or other conditions.

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| 1  | Backman *et al.* (1975) | • Design: The amino acid pattern in plasma was studied in a reference group (n=26) and in three groups of massive obese subjects (n=9, 8, and 9 respectively) before and at intervals after jejuno-ileostomy. | • The concentrations of lysine, tyrosine, cystine, and glutamic acids were higher, and aspartamin, glutamine, serine, and glycine were lower than in the reference group.  
• During the post-operative period the amino acid pattern changed significantly with serine, glycine, and taurine increased and valine, lysine, leucine, tryptophan, threonine, cystine, and citrulline decreased.  
• The amino acid pattern in the obese group with the longest post-operative observation time and a stable body weight differed significantly from that in the reference group only with regard to a low valine concentration and high concentration of taurine and glutamic acid. |
| 2  | Müller *et al.* (1983)   | • Sample: 6 patients - infusing 0.3 mg/24 h of exogenous glucagon                             | • In six normal subjects the same infusion reduced significantly (p < 0.05) plasma alanine, asparagine, glutamate, glutamine, glycine, proline, serine, threonine, arginine, ornithine, lysine and tyrosine  
• This particular glucagon sensitivity of duodenopancreatectomized patients suggests that glucagon deficiency is the cause of their hyperaminacidaemia. |
| 3  | Jeevanandam *et al.* (1991) | • Sample: 10 obese and 10 non-obese traumatized patients  
• Plasma levels of free amino acids in the early flow phase of injury when subjects were receiving maintenance fluids without calories or nitrogen | • Obese controls showed an increase in valine, leucine, isoleucine, and glutamic acid levels, and a decrease in glycine, tryptophan, threonine, histidine, taurine, citrulline, and cystine levels compared with lean controls.  
• Hypoaminoacidemia was equally seen in traumatized obese and non-obese patients, and it was mainly due to a 24% decrease in nonessential amino acids.  
• Essential amino acid levels were the same in all groups. |
| 4  | Sandstrom *et al.* (2003) | • Sample: Serum L-arginine and L-citrulline and urinary nitrite/nitrate concentrations 1 to 3 days after the onset of symptoms in 11 patients with gallstone pancreatitis, 10 patients with alcoholic pancreatitis, and 6 patients with idiopathic pancreatitis. 13 healthy control blood donors, 9 patients fasting before hernia operations, 8 patients with acute cholecystitis, and 9 alcoholic subjects but no pancreatitis.  
• Serum arginine and citrulline concentrations were measured with high performance liquid chromatography, and urinary nitrite/nitrate spectrophotometrically. | • Patients with acute pancreatitis had lower serum L-arginine and L-citrulline concentrations than controls. |
| 5  | Sandstrom *et al.* (2008) | • Design: Serum amino acid spectrum was measured daily for five days and after recovery six weeks later in 19 patients admitted to the hospital for acute pancreatitis. | • These patients had abnormal levels of most amino acids including arginine, citrulline, glutamine and glutamate.  
• Phenylalanine and glutamate were increased, while arginine, citrulline, ornithine and glutamine were decreased compared to levels after recovery. |
Table 2.6 (Continued)

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| 6  | Thibault et al. (2008); Thibault et al. (2009) | • Sample: 20 morbidly obese patients operated by Roux-en-Y gastric bypass (17 women, 47 ± 12 years, BMI, 53.3±11.3 kg/m²)  
   • Body composition determined by single-frequency bioelectrical impedance analysis. Blood testing.  
   • Plasma concentrations of 20 amino acids including citrulline were available for only 7 patients. | • Plasma citrulline (53.6 ± 16.0 μmol/L) and other amino acids, except cysteine, did not differ from normal values                                                                 |
| 7  | Luiking et al. (2009)    | • Aim: To compare arginine and citrulline metabolism in septic patients and nonseptic control patients in an intensive care unit and in healthy control subjects.  
   • Sample: 10 patients with septice shock, 7 critically ill control patients, and 16 healthy elderly subjects | • Whole-body citrulline production was significantly lower in septic patients (4.5 ± 2.1 μmol/kg/h) than in intensive care control patients (10.1 ± 2.9 μmol/kg/h, p < 0.01) and in healthy control subjects (13.7 ± 4.1 μmol/kg/h, p < 0.001)  
   • Citrulline production is severely low in patients with sepsis and is related to diminished de novo arginine and nitric oxide production |
| 8  | Peters et al. (2009)     | • Aim: To assess citrulline generation test reference values in 14 stable intensive care patients with respiratory failure with normal renal function and able to tolerate enteral nutrition  
   • Amino acid analysis was performed using reverse phase high performance liquid chromatography  
   • 8 females, 6 males, mean age 60.2 years and BMI 27.2 kg/m² | • The incremental area under the curve at 90 minutes during the test following enteral glutamine was 5,807,437 mmol/L/min for venous and 6,807,507 mmol/L/min for arterial citrulline sampling  
   • Performing the test with intravenously administered glutamine resulted in an area of 7,707,235 mmol/L/min for venous and 9,297,223 mmol/L/min for arterial citrulline sampling  
   • Positive correlation between venous and arterial citrulline sampling in enteral (r = 0.96, p < 0.0001) and intravenous glutamine (r = 0.91, p < 0.0001) |
| 9  | Crenn et al. (2010)      | • Aim: To investigate in septic shock patients with multi-organ failure plasma citrulline pharmacokinetics, associated parameters and pro tumour necrosis factor alpha anti-interleukin-10-inflammatoty plasma cytokines  
   • Two groups (n = 16,7 males, age 63 ± 12 years) were selected: survivors (n = 8), deceased patients (n = 8) | • Citrulline decreased during day 0 (29 ± 10 vs18 ± 6 μmol/L, p < 0.05) in most patients  
   • Citrulline remained < 10 μmol/L in 2 patients of the deceased group whereas a transient citrulline <10 μmol/L was noted in 2 survivors. Citrulline normalised on day 7 in 5 survivors and 1 deceased patient (p = 0.10)  
   • Citrulline was negatively correlated with C-Reactive protein (r = 0.31, p < 0.01) but positively with glutamine, arginine and creatinine (r = 0.95, 0.92, 0.25, p< 0.05)  
   • No significant correlation was found between citrulline and albumin, tumour necrosis factor alpha, and interleukin-10 |
| 10 | Pan et al. (2010)        | • Sample: 32 patients with acute pancreatitis onset within 7 days  
   • Severity of disease and gut dysfunction on admission, on day 7, and day 3 of enteral nutrition  
   • Serum levels of intestinal fatty acid binding protein, citrulline, and C-reactive protein (CRP) and the lactulose and mannitol absorption ratio in urine were measured in parallel | • Intestinal fatty acid binding protein increased on admission and in severe attacks  
   • All patients: ↑ gut dysfunction score, C-reactive protein, urine level of lactulose and mannitol absorption ratio; ↓ citrulline  
   • Positive correlation noted between intestinal fatty acid binding protein and gut dysfunction score, Acute Physiology and Chronic Health Evaluation II score, C-reactive protein and intensive care stay  
   • Negative correlation noted between intestinal fatty acid binding protein and citrulline |
| 11 | Piton et al. (2010)      | • Design: Prospective observational p67 patient without small bowel disease and without chronic renal failure consecutively admitted to a single intensive care unit  
   • Plasma citrulline concentrations were studied at admission, 12, 24, 48 hours, and the 7th day after admission | • 1st day: mean citrulline decreased from 18.8 to 13.5 μmol/L  
   • Low plasma citrulline at 24 hours was associated with low plasma glutamine (p = 0.01) and arginine (p = 0.04), high plasma C-reactive protein (p = 0.008), nosocomial infection rate (p = 0.03), and 28-day mortality (p = 0.02)  
   • Multivariate analysis: plasma citrulline ≤ 10 μmol/L at 24 hours and Sequential Organ Failure Assessment score ≥ 8 at 24 hours had higher 28-day mortality (odds ratio 8.7, 15.1, respectively) |
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| 12  | Través et al. (2010)      | • Sample: 28 patients who underwent subtotal gastrectomy or hemicolecotomy and were placed on short-term parenteral nutrition  
• Design: Assigned on a Parenteral-Oral (4-day parenteral nutrition and 4-day oral, n = 8) or a Parenteral-Only (7-day parenteral nutrition, n = 20) nutritional regime | • Pre-operative citrulline values were within range with those of a western population.  
• On day 4 in the Parenteral-Oral regime, citrulline levels were 60% lower than pre-operative levels  
• When enteral feeding was resumed, citrulline rose and was close to pre-operative values on day 8  
• In the Parenteral-Only regime the parenteral nutrition solution composition had no influence on the citrulline |
| 13  | van Noord et al. (2011)   | • Sample: Consecutive patients suspected of chronic gastrointestinal ischaemia (n = 40), healthy subjects (n = 9)  
• Blood samples for analysis of intestinal fatty acid-binding protein, D-dimer, lactate dehydrogenase, leucocyte counts, C-reactive protein, and L-lactate were drawn before and after a standard meal.  
• Intestinal mucosal injury was assessed with glutamine, citrulline and arginine in blood samples and compared to a sugar absorption test | • Ischaemia diagnosed in 32 patients  
• No difference noted in any parameter between patients with and without ischaemia  
• L-lactate was increased in ischaemia patients compared to non-ischaemia patients.  
• In ischaemia patients, D-dimer levels showed a significant elevation post-prandially compared to baseline. |
| 14  | Verdam et al. (2011)      | • Aim: To investigate the relation between plasma markers of small intestinal function and chronic hyperglycaemia  
• Sample: Cross-sectional observational study of 40 severely obese subjects with chronic hyperglycaemia and 30 severely obese subjects without chronic hyperglycaemia who were indicated for bariatric surgery.  
• Measurement of plasma levels of citrulline, intestinal fatty acid binding protein, glucagon-like peptide-2, glycated haemoglobin HbA1c | • Plasma citrulline and intestinal fatty acid binding protein levels were significantly elevated in chronic hyperglycaemia compared to normal HbA1c: (Citrulline: 35 ± 2.1 vs 26 ± 1.4 μmol/L, p = 0.001; intestinal fatty acid binding protein: 140 ± 22 vs 69 ± 14 pg/mL, p = 0.001)  
• Plasma citrulline and intestinal fatty acid binding protein correlated with HbA1c (r = 0.30, 0.33, p < 0.05, respectively).  
• Intestinal fatty acid binding protein to citrulline ratio was higher in subjects with elevated HbA1c (4.0 vs 3.1, p = 0.03)  
• Glucagon-like peptide-2 was not related to citrulline or intestinal fatty acid binding protein (p > 0.05) |
| 15  | Lundy et al. (2012)       | • Design: Observations of serial plasma citrulline levels in a severely burned adult who ultimately died from non-occlusive mesenteric ischaemia leading to full-thickness small bowel necrosis | • Decrease of citrulline around settings of ischaemia and increase of lactate |
| 16  | Noordally et al. (2012)   | • Aim: Prospective observational single-centre controlled study  
• Inclusion criteria: intensive care stay over 48 hours  
• Plasma citrulline: low (0-15 μmol/L), medium (16-35 μmol/L), and high (>36 μmol/L) | • Mean citrulline: 21.7 ± 13.1 μmol/L  
• Patients with intestinal dysfunction had low plasma citrulline level < 15 μmol/L (p = 0.014)  
• No correlations noted between C-reactive protein, albumin, prealbumin, renal failure, inotrope use, Sequential Organ Failure Assessment score, Acute Physiology and Chronic Health Evaluation II score and citrulline |
| 17  | Grimaldi et al. (2013)    | • Sample: 21 patients following resuscitation after cardiac arrest  
• Urinary intestinal fatty acid-binding protein, plasma citrulline, whole blood endotoxin were measured at admission, days 1-3 and 6  
• Kinetics of release and the relationship between intestinal fatty acid-binding protein, citrulline and endotoxin values | • Lowest median of citrulline was attained at day 2 (11 vs 24 μmol/L at admission, p = 0.01) and normalised at day 6 (21 μmol/L)  
• Highest endotoxin level was negatively correlated lowest plasma citrulline levels (r² = 0.55, p < 0.001) |
<table>
<thead>
<tr>
<th>No.</th>
<th>Authors</th>
<th>Settings (sample and design)</th>
<th>Main Results</th>
</tr>
</thead>
</table>
| 18  | Kao et al. (2013) | - Aim: To investigate how sepsis affects glutamine metabolism, including its conversion to citrulline, by measuring glutamine and citrulline flux, fractional splanchnic extraction of glutamine and leucine, and the contribution of glutamine nitrogen to citrulline in septic patients and healthy controls  
Sample: 8 patients with severe sepsis and 10 healthy controls were given primed, constant intravenous infusion of $[^3]$H-citrulline and sequential administration of intravenous and enteral $[^{15}$N]glutamine and $[^{13}$C]leucine in the post-absorptive state | - Compared with healthy controls, septic patients had a significantly lower whole body citrulline flux and plasma concentration, higher endogenous leucine flux, and higher glutamine clearance  
- The majority of the $[^3]$N label transferred from glutamine to citrulline was found at the α-position  
- Lower glutamine plasma concentrations in sepsis were a result of increased glutamine clearance  
- Despite adequate splanchnic uptake of glutamine, there is decreased production of citrulline, suggesting a defect in the metabolic conversion of glutamine to citrulline, decreased uptake of glutamine by the enterocyte but increased uptake by the liver, and/or shunting of glutamine to other metabolic pathways |
| 19  | Piton et al. (2013) | - Design and Sample: 103 intensive care patients, prospective observational study  
- Inclusion criteria: 18 years old or older; expected intensive care stay over 48 hours, without pregnancy, chronic small bowel disease, or chronic renal failure  
- Plasma intestinal fatty acid-binding protein, citrulline concentrations, and variables relating to prognosis and treatment, were measured on admission | - Intestinal fatty acid-binding protein elevation on admission was associated with catecholamine support, higher lactate concentration, higher Sequential Organ Failure Assessment score, and higher international normalized ratio (p < 0.001)  
- Plasma citrulline concentration ≤ 10 μmol/L on admission was associated with higher intra-abdominal pressure, higher plasma C-reactive protein concentration, and more frequent antibiotic use (p < 0.005)  
- No correlation between plasma levels of intestinal fatty acid-binding protein and citrulline  
- On admission, Sequential Organ Failure Assessment score ≥ 12, plasma citrulline ≤ 12.2 μmol/L, and plasma intestinal fatty acid-binding protein concentration ≥ 355 pg/mL were associated with higher 28-day mortality (odds ratio 4.39, 5.17, 4.46, respectively) |
| 20  | Ware et al. (2013) | - Sample: Plasma citrulline, arginine and ornithine levels and nitrate/nitrite were measured at baseline in 135 patients with severe sepsis  
- Acute respiratory distress syndrome was diagnosed by a consensus definition | - Plasma citrulline levels:  
- Below normal in all patients: median 9.2 (5.2–14.4) μmol/L  
- Acute respiratory distress syndrome vs non-acute respiratory distress syndrome: 6.0 (3.3–10.4) vs 10.1 (6.2–16.6) μmol/L, p = 0.002  
- The rate of acute respiratory distress syndrome was 50% in the lowest citrulline quartile compared to 15% in the highest citrulline quartile (p = 0.002)  
- In multivariable analyses, citrulline levels were associated with acute respiratory distress syndrome after adjustment for covariates including illness severity |
| 21  | Alekseeva and Sal’nikov (2014) | - Sample: 27 critical condition patients (15 females, age 70 ± 14 years  
- On admission to intensive care, plasma glutamine, citrulline, glutamic acid (liquid chromatography), relative duodenal and jejunal electrical activity were measured | - No increase noted in plasma glutamine, citrulline or glutamic acid  
- Worst prognosis was observed when citrulline was ≤ 10 μmol/L and signified decrease in proximal small intestine relative electrical activity |
| 22  | Carswell et al. (2014) | - Sample: obese controls (BMI > 30 kg/m², n = 7), adjustable gastric banding (n = 6), Roux-en-Y gastric bypass (n = 7), biliopancreatic diversion with duodenal switch (n = 5).  
- Measurements: oro-caecal transit time, fasting plasma citrulline, 3 days of faecal elastase 1, calprotectin, fatty acids | - No difference in oro-caecal transit time (p = 0.935) or citrulline levels (p = 0.819)  
- Faecal calprotectin was elevated post-Roux-en-Y gastric bypass vs obese (p = 0.016) and faecal elastase 1 was decreased post-Roux-en-Y gastric bypass vs obese (p = 0.002) |
Table 2.6  (Continued)

<table>
<thead>
<tr>
<th>No</th>
<th>Authors</th>
<th>Settings (sample and design)</th>
<th>Main Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Piton et al.</td>
<td>Sample: 69 patients with cardiac arrest of both cardiac and hypoxic origin admitted to intensive care</td>
<td>On admission, citrulline was low in 65 % and plasma intestinal fatty acid-binding protein was high in 82 %</td>
</tr>
<tr>
<td></td>
<td>(2015a)</td>
<td>Design: Prospective, observational, single-centre study, evaluating plasma citrulline and intestinal fatty acid-binding protein concentrations on admission and after 24 hours</td>
<td>At 24 hours, citrulline was low in 82 % and intestinal fatty acid-binding protein was normal in 60 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Comparison of the variables according to 28-day Cerebral Performance Category score of 1-2 (good neurological outcome) vs 3-5 (poor neurological outcome)</td>
<td>Patients with a poor neurological outcome had a lower plasma citrulline concentration and a higher intestinal fatty acid-binding protein on admission</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>Multivariate analysis: plasma citrulline levels ≤ 13.1 μmol/L and intestinal fatty acid-binding protein &gt; 260 pg/mL were independently associated with a poor neurological outcome (odds ratio 21.9, 13.6, respectively)</td>
</tr>
<tr>
<td>24</td>
<td>Piton et al.</td>
<td>Aim: To examine whether catecholamines in critically ill patients may be associated with enterocyte damage</td>
<td>Plasma intestinal fatty acid-binding protein was higher among patients receiving catecholamine vs controls</td>
</tr>
<tr>
<td></td>
<td>(2015b)</td>
<td>Design: Prospective observational study.</td>
<td>In patients receiving catecholamines, a dose of 0.48 γ/kg/min or more on admission was associated with a higher intestinal fatty acid-binding protein concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sample: Critically ill patients requiring epinephrine and/or norepinephrine on admission to intensive care (n = 60). Controls not receiving catecholamines (n = 27)</td>
<td>Sepsis-related Organ Failure Assessment score &gt; 11 and plasma intestinal fatty acid-binding protein more than 524 pg/mL on admission were independently associated with 28-day mortality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measurement on admission: plasma intestinal fatty acid-binding protein, plasma citrulline, abdominal perfusion pressure, and variables relating to prognosis and treatment</td>
<td>Citrulline was not associated with catecholamine dose but was generally low: median 14.7 (8.8-27.9).</td>
</tr>
<tr>
<td>25</td>
<td>Poole et al.</td>
<td>Sample: Prospective observational study, 15 healthy, 20 critically ill subjects</td>
<td>Healthy subjects vs critically ill patients: citrulline 26.5 vs 15.2 μmol/L, p &lt; 0.01; glucose absorption 79.7 vs 61.0 mmol/L/240 min, p = 0.05</td>
</tr>
<tr>
<td></td>
<td>(2015)</td>
<td>Fasting plasma citrulline concentrations were assayed in blood samples immediately prior to the administration of a liquid test meal (1 kcal/ml; containing 3 g of 3-O-methylglucose) that was infused directly into the small intestine</td>
<td>No relationship between fasting citrulline concentration and subsequent glucose absorption was noted (r = 0.28; p = 0.12)</td>
</tr>
</tbody>
</table>
Table 2.7. Common causes of short bowel syndrome (Nightingale and Woodward, 2006; Pironi et al., 2016; Pironi et al., 2018).

<table>
<thead>
<tr>
<th>Jejunum-colon</th>
<th>Jejunostomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s disease</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>Mesenteric ischaemia</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Irradiation</td>
</tr>
<tr>
<td>Small bowel volvulus</td>
<td>Mesenteric ischaemia</td>
</tr>
<tr>
<td>Adhesions</td>
<td>Desmoid</td>
</tr>
</tbody>
</table>

Table 2.8. Metaregression results of the correlation of citrulline with small bowel length with four potential sources of heterogeneity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95% CI)</th>
<th>SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male %</td>
<td>-0.0196 (-0.1155, 0.0763)</td>
<td>0.03</td>
<td>0.562</td>
</tr>
<tr>
<td>Age</td>
<td>0.0500 (-0.1267, 0.2268)</td>
<td>0.06</td>
<td>0.434</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.0088 (-0.1593, 0.1416)</td>
<td>0.05</td>
<td>0.864</td>
</tr>
<tr>
<td>Mean citrulline concentraion</td>
<td>-0.0779 (-0.3997, 0.2439)</td>
<td>0.10</td>
<td>0.497</td>
</tr>
<tr>
<td>Mean small bowel length</td>
<td>-0.0266 (-0.1043, 0.0510)</td>
<td>0.02</td>
<td>0.355</td>
</tr>
<tr>
<td>Constant</td>
<td>3.7196 (-5.4980, 12.9372)</td>
<td>2.90</td>
<td>0.289</td>
</tr>
</tbody>
</table>

Restricted maximum likelihood estimate of between-study variance: $\tau^2 = 0.8593$

% residual variation due to heterogeneity: $I^2 = 98.9\%$

Proportion of between-study variance explained: Adjusted $R^2 = 0\%$

Joint test for all covariates: Model $F(5,3) = 0.34$

With Knapp-Hartung modification: $p > F = 0.862$
Table 2.9. Diagnostic accuracy data regarding studies investigating short bowel syndrome.

<table>
<thead>
<tr>
<th>Study</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>Citrulline cut-off level (μmol/L)</th>
<th>AUC (ROC)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Groups under comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crenn et al. (2000)</td>
<td>34</td>
<td>2</td>
<td>3</td>
<td>18</td>
<td>20</td>
<td>92 %</td>
<td>91 %</td>
<td></td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Rhoads et al. (2005)</td>
<td>13</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>20</td>
<td>0.91 (0.79, 1.00)</td>
<td>81 %</td>
<td>100 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Peters et al. (2007c)</td>
<td>8</td>
<td>4</td>
<td>22</td>
<td>14</td>
<td>20</td>
<td>0.50 (0.30, 0.71)</td>
<td>25 %</td>
<td>77 %</td>
<td>SBS vs Controls</td>
</tr>
<tr>
<td>Papadia et al. (2007)</td>
<td>22</td>
<td>4</td>
<td>5</td>
<td>24</td>
<td>21</td>
<td>0.87</td>
<td>83 %</td>
<td>87 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Parekh et al. (2008)</td>
<td>30</td>
<td>21</td>
<td>0</td>
<td>28</td>
<td>20</td>
<td>0.82 (0.71, 0.93)</td>
<td>100 %</td>
<td>57 %</td>
<td>SBS vs Controls</td>
</tr>
<tr>
<td>Santarpia et al. (2008)</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>10</td>
<td>0.65 (0.43, 0.88)</td>
<td>100 %</td>
<td>9 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Fitzgibbon et al. (2009)</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>15</td>
<td>0.88 (0.75, 1.00)</td>
<td>89 %</td>
<td>78 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Bailly- Botuha et al. (2009)</td>
<td>20</td>
<td>9</td>
<td>1</td>
<td>3</td>
<td>20</td>
<td>0.46 (0.25, 0.67)</td>
<td>95 %</td>
<td>25 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Diamanti et al. (2010)</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>20</td>
<td>0.65 (0.78, 1.00)</td>
<td>64 %</td>
<td>93 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Pironi et al. (2011)</td>
<td>25</td>
<td>25</td>
<td>2</td>
<td>20</td>
<td>20</td>
<td>0.87</td>
<td>50 %</td>
<td>91 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Raphael et al. (2011)</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>20</td>
<td>0.65 (0.78, 1.00)</td>
<td>64 %</td>
<td>93 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Diamanti et al. (2011b)</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>13</td>
<td>10</td>
<td>0.90 (0.70, 1.00)</td>
<td>64 %</td>
<td>93 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Pironi et al. (2012)</td>
<td>33</td>
<td>0</td>
<td>34</td>
<td>26</td>
<td>14</td>
<td>0.87</td>
<td>49 %</td>
<td>100 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Suzuki et al. (2012)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>15</td>
<td>0.85 (0.78, 0.92)</td>
<td>31 %</td>
<td>93 %</td>
<td>HPN dependency</td>
</tr>
<tr>
<td>Amiot et al. (2013)</td>
<td>38</td>
<td>10</td>
<td>86</td>
<td>134</td>
<td>20</td>
<td>0.87</td>
<td>55 %</td>
<td>83 %</td>
<td>SBS vs Controls</td>
</tr>
<tr>
<td>Pinto Costa et al. (2013)</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>25.5</td>
<td>0.67 (0.46, 0.88)</td>
<td>55 %</td>
<td>83 %</td>
<td>SBS vs Controls</td>
</tr>
</tbody>
</table>
Table 2.10. Results of diagnostic meta-analysis regarding sensitivity and specificity in patients with all conditions and only short bowel syndrome.

<table>
<thead>
<tr>
<th></th>
<th>All conditions (26 studies)</th>
<th>Short bowel syndrome (16 studies)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient (95% CI)</td>
<td>SE</td>
</tr>
<tr>
<td><strong>Bivariate Model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected value of logit[Sensitivity]</td>
<td>1.37 (0.82, 1.92)</td>
<td>0.28</td>
</tr>
<tr>
<td>Expected value of logit[Specificity]</td>
<td>1.63 (1.19, 2.06)</td>
<td>0.22</td>
</tr>
<tr>
<td>Variance of logit[Sensitivity]</td>
<td>1.57 (0.77, 3.19)</td>
<td>0.57</td>
</tr>
<tr>
<td>Variance of logit[Specificity]</td>
<td>0.82 (0.37, 1.85)</td>
<td>0.34</td>
</tr>
<tr>
<td>Correlation between logits</td>
<td>-0.55 (-0.84, -0.02)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>HSROC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\lambda)</td>
<td>3.08 (2.51, 3.64)</td>
<td>0.29</td>
</tr>
<tr>
<td>(\theta)</td>
<td>-0.37 (-0.89, 0.14)</td>
<td>0.26</td>
</tr>
<tr>
<td>(\beta)</td>
<td>-0.32 (-0.82, 0.17)</td>
<td>0.25</td>
</tr>
<tr>
<td>(\sigma^2\alpha)</td>
<td>1.03 (0.37, 2.82)</td>
<td>0.53</td>
</tr>
<tr>
<td>(\sigma^2\theta)</td>
<td>0.88 (0.45, 1.72)</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>80 % (69%, 87%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Specificity</td>
<td>84 % (77%, 89%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Diagnostic odds ratio</td>
<td>20.03 (11.55, 34.72)</td>
<td>5.62</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>4.85 (3.47, 6.80)</td>
<td>0.84</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.24 (0.16, 0.37)</td>
<td>0.05</td>
</tr>
<tr>
<td>Inverse negative likelihood ratio</td>
<td>4.13 (2.72, 6.25)</td>
<td>0.87</td>
</tr>
<tr>
<td><strong>Model characteristics</strong></td>
<td>Covariance between estimates of Expected value of logit[Sensitivity] and Expected value of logit[Specificity]: -137.82452 Log likelihood = -0.0247892</td>
<td>Covariance between estimates of Expected value of logit[Sensitivity] and Expected value of logit[Specificity]: -0.06415 Log likelihood = -79.102622</td>
</tr>
</tbody>
</table>
2.6 Figures

463 studies identified through database searching

Abstracts of records screened

209 of full-text articles assessed for eligibility

131 studies were trials or case reports or case series investigating clonidine and diarrhoea and were analysed in the systematic review

63 studies included in the meta-analysis

254 records excluded for irrelevant content (not related to the inclusion criteria)

78 of full-text articles excluded (reviews, animal studies, experimental studies, irrelevant topics)

68 studies not included in the meta-analysis because no data could be extracted

Figure 2.1. Flow chart for systematic review.
Figure 2.2. Risk of bias graph: review authors’ judgements about each risk of bias item presented as percentages across all included studies (all studies).

Figure 2.3. Risk of bias summary: review authors’ judgements about each risk of bias item for each included study.
Figure 2.4. Forest plot with overall mean difference of patients with a condition against controls (30 studies) (overall and subgroups).
<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Total</th>
<th>Correlation IV, 95% CI</th>
<th>Correlation IV, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Miami Group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pappas et al. 2001</td>
<td>49</td>
<td>-0.59 [-0.75, -0.37]</td>
<td></td>
</tr>
<tr>
<td>David et al. 2006</td>
<td>37</td>
<td>-0.42 [-0.65, -0.11]</td>
<td></td>
</tr>
<tr>
<td>David et al. 2007</td>
<td>2135</td>
<td>-0.41 [-0.44, -0.37]</td>
<td></td>
</tr>
<tr>
<td>Ruiz et al. 2010</td>
<td>10352</td>
<td>-0.97 [-0.97, -0.97]</td>
<td></td>
</tr>
<tr>
<td>Hibi et al. 2012</td>
<td>13499</td>
<td>-0.98 [-0.98, -0.98]</td>
<td></td>
</tr>
<tr>
<td><strong>Mount Sinai Group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gondolesi et al. 2004</td>
<td>145</td>
<td>-0.43 [-0.55, -0.28]</td>
<td></td>
</tr>
<tr>
<td>Gondolesi et al. 2006</td>
<td>403</td>
<td>-0.31 [-0.40, -0.22]</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.5.** Forest plot of Intestinal Transplantation studies (Citrulline concentrations correlations with rejection) – without meta-analysis due to severe heterogeneity.
Figure 2.6. Forest plot of studies measuring plasma citrulline in short bowel syndrome. Correlation of citrulline with small bowel length.

<table>
<thead>
<tr>
<th>Study</th>
<th>Total</th>
<th>Weight</th>
<th>Correlation IV, Random, 95% CI</th>
<th>Correlation IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crenn et al. 2000</td>
<td>57</td>
<td>4.7%</td>
<td>0.83 [0.73, 0.90]</td>
<td></td>
</tr>
<tr>
<td>Kabrt et al. 2003</td>
<td>20</td>
<td>4.5%</td>
<td>0.37 [-0.08, 0.70]</td>
<td></td>
</tr>
<tr>
<td>Pita et al. 2003</td>
<td>13</td>
<td>4.3%</td>
<td>0.77 [0.38, 0.93]</td>
<td></td>
</tr>
<tr>
<td>Gong et al. 2005</td>
<td>22</td>
<td>4.5%</td>
<td>0.82 [0.61, 0.92]</td>
<td></td>
</tr>
<tr>
<td>Rhoads et al. 2005</td>
<td>45</td>
<td>4.7%</td>
<td>0.47 [0.20, 0.67]</td>
<td></td>
</tr>
<tr>
<td>Luo et al. 2007</td>
<td>24</td>
<td>4.6%</td>
<td>0.47 [0.08, 0.73]</td>
<td></td>
</tr>
<tr>
<td>Nion–Larmurier et al. 2007</td>
<td>23</td>
<td>4.6%</td>
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Total (95% CI) 986 100.0% 0.67 [0.39, 0.84]

Heterogeneity: Tau² = 0.91; Chi² = 756.93, df = 21 (P < 0.01); I² = 97%
Test for overall effect: Z = 3.92 (P < 0.01)
Figure 2.7. Funnel plot of short bowel syndrome studies. Correlation of citrulline with small bowel length – there is asymmetry in the plot indicating publication bias.
Figure 2.8. Forest plots of short bowel syndrome correlations with small bowel length – subgroup analyses by (A) continent, (B) country, (C) patient type, and (D) citrulline measurement method.
Figure 2.9. Funnel plots. A. Mean citrulline levels: All conditions vs controls (30 studies). No asymmetry seen. B. Mean citrulline levels: SBS vs controls. No asymmetry observed. C. Mean citrulline levels: CeD/Enteropathy vs controls. No asymmetry observed. D. Mean citrulline levels: PN dependent vs PN independent patients. No asymmetry observed. E. Citrulline levels with disease severity in all conditions (28 studies). No asymmetry seen. F. Citrulline levels with disease severity in mucositis after chemoradiation. No asymmetry observed.
Figure 2.10. Forest plots. (A) Mean differences of citrulline levels between PN dependent and independent SBS patients. (B) Mean increase of citrulline levels after treatment with teduglutide vs placebo in SBS patients. (C) Mean increase of citrulline levels after treatment with teduglutide vs baseline in SBS patients. (D) Mean difference of citrulline levels in coeliac disease patients who had received GFD treatment vs those who had not.
### Short bowel syndrome

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**Figure 2.11.** Forest plots of sensitivity and specificity in all patient conditions (26 studies).
Figure 2.12. Summary ROC curve for diagnostic accuracy in SBS patients.
Figure 2.13. Forest plot with correlation coefficients with severity in all available studies (28 studies).
Figure 2.14. Summary ROC curve for all studies of diagnostic accuracy (26 studies).
Figure 2.15. Forest plot with correlation coefficients of citrulline levels with enteral absorption (14 studies).
Chapter 3
Pharmacodynamic Study of Post-absorptive Plasma Citrulline after Oral Amino Acid Challenges

3.1 Introduction

Until now, it has been shown that citrulline is a significant diagnostic marker in intestinal failure patients. However, it is important for physicians to have better diagnostic tools at their disposal in order to tackle this disease. The studies mentioned in Chapter 2 take static measurements of citrulline in certain clinical conditions. These are mostly observational and show a static correlation of citrulline and intestinal function. There are no studies examining how amino acid challenges could reflect enterocyte function.

There is a central problem with the past data that although predictive for populations, the predictive value of a given citrulline level for an individual patient is poor i.e. not a sufficiently bimodal distribution. Pharmacodynamic challenge tests are an important step in diagnostic test development. Following paradigms from endocrinology and psychopharmacology, where challenge tests are integral to research and clinical practice (e.g. short synacthen test, glucose challenge test etc.), the Guideline for Good Clinical Practice of the International Conference on Harmonization may possibly be of great help in standardizing the development of challenge tests (EMEA, 1997; Gijsman et al., 2004). Challenge tests are particularly useful in situations where measured markers are part of a physiological axis or pathway and challenging a member of this pathway is useful to clarify where the pathway might be failing.

This gap in nutrition literature is what this chapter addresses. I give the outline of a certain study design executed in order to explore the pharmacodynamic diagnostic properties of citrulline.

Study: “Randomised cross-over, single-site study on the bioavailability of Citrulline after an oral bolus of Citrulline or Glutamine or Arginine or 3-Methylhistidine or placebo in healthy subjects”.

The present chapter gives a description of the actual design of this pharmacodynamics study with sections dedicated to trial design, sample size, and
laboratory analysis of samples. The sections have theoretical descriptions in between, where topics on sample size, crossover designs, randomisation, carryover and washout effects are discussed. First, I begin with a theoretical discussion on the design of experiments.

### 3.2 Design of Experiments: A (Theoretical) Primer

Before a detailed description of the clinical study is given, basic concepts regarding design of experiments are outlined. Design of experiments is a special chapter in statistics, which one can easily access by reading the books by Cox and Reid (2000), Giesbrecht and Gumpertz (2004) or Cochran and Cox (1957) among others. In this section, I intend to give some basics in the design of experiments and to elaborate deeper in crossover designs.

Thus, to begin with it is necessary to mention that statistics can have the greatest impact on the quality of an experimental study while in the planning stage. The feasibility of any experiment is constrained by limitations on time, equipment, funds, and ethical considerations. Ethical constraints range from sample size calculations which is related to use of resources (e.g. large experiments use more resources than needed but small experiments waste resources because they fail to give answers) to serious considerations, such as causing pain and suffering to living organisms or even to fellow human beings, or harming the environment. The goals of statistical experimental design are to provide tools that help to ensure that the experiment will be capable of providing answers to the research questions posed and that it will provide the information as efficiently as possible using the available resources and respecting all ethical constraints. An experimental plan that does not respect ethical constraints is not a good plan.

The statistician is rarely the lead investigator in scientific investigations, but statistics has some valuable insights and tools to offer to the process of designing experiments. Questions that come up when an experiment is designed involve:

1. How many participants should be examined? How much replication should there be?

2. How does one construct the experiment to provide as broad a basis for inference as possible?
3. How does one take into account the natural variability among experimental units?

4. How should treatments be allocated to the entities being studied?

5. How is the statistical analysis to be performed?

For these questions, there are four key principles of design of experiments that need to be emphasized:

(1) representativeness, (2) randomisation, (3) replication, and (4) error control or blocking. These ideas are fundamental to making an experiment useful for providing answers to the research questions. Representativeness, proper randomisation, and sufficient replication and error control are the grounds upon inferences can be made without bias and with enough precision to be meaningful. Randomisation provides insurance that unanticipated factors do not accidentally introduce unwanted biases into experimental results. Replication and error control are necessary to ensure that results are generally true and not just the product of the special circumstances of a particular run (Cochran and Cox, 1957; Giesbrecht and Gumpertz, 2004).

The process of designing an experiment has three parts. First, it is necessary to understand the objectives and constraints clearly, state what quantities are to be estimated, and identify what hypotheses are to be tested. The comment that a problem is too complex, or involves too many ethical or financial constraints for a properly designed experiment, reflects poor understanding of the experimental process. The second task is to consider possible designs that are tailored to the aims of the experiment and yet satisfy all ethical, physical, and financial constraints. The final part of the process is to evaluate the proposed design to establish that the objectives can be achieved, that the estimates are likely to have sufficient precision, and that any proposed tests of hypotheses have adequate power. Outlining the statistical analysis before the experiment is actually conducted is always a good idea. It allows the investigator to see, clearly, what can be estimated and what hypotheses can be tested before any data are collected. It is not uncommon to find an experiment that appears reasonable and yet on closer examination fails to provide estimates of important quantities or does not allow testing the hypotheses of interest because of insufficient replication or flawed allocation of treatments.
Outlining the statistical analysis during the planning stage helps prevent such mistakes (Cochran and Cox, 1957; Giesbrecht and Gumpertz, 2004).

3.2.1 Replication and Handling Unexplained Variability

The precision (or inversely, the variability) of estimates and the power of hypothesis tests depend on the amount of replication and the magnitude of the unexplained variation among experimental units. In general, the more replication, the more precise the estimates (Cox and Reid, 2000). The amount of replication and the pattern of how many units are assigned to each treatment are things that the investigator can control. If an experiment is not replicated properly, it may not be possible to estimate the precision at all. It is important to know how precise estimates are in order to have some idea of the likelihood or assurance that an estimate falls within reasonable distance of the true (but unknown) value.

In some cases it is possible to reduce unexplained variation by using the technique of blocking (Bailey, 2008). This does not reduce the unexplained variation per se, but it assigns treatments to experimental units in such a way that the unexplained variation is screened out before making comparisons among the treatments (Gates, 1995).

Finally, the unexplained variation can often be reduced by improving the model. This can be done by building in additional explanatory variables, also called covariates, or by changing the form of the model. The decision to measure additional variables should be made in the planning stages before the experiment is executed (Montgomery, 2009).

Of the three methods described: (1) improving experimental technique, (2) blocking, and (3) improving the model, the first is usually up to the subject-matter scientist, and the latter two are statistical devices. The goal of all three is to reduce the unexplained variation so that the effects of treatments stand out from the background noise and can be identified more easily (Giesbrecht and Gumpertz, 2004).

3.2.2 Randomisation: Why and How

The original notion of randomization is usually credited to Fisher (1935) and is accepted by most experimenters. Unfortunately, not all practice it. Too often,
haphazard assignment of treatments to experimental units is the practice used instead of true randomization. The two are not the same. Another common error is to assume that any randomization scheme that assigns each treatment to every experimental unit equally frequently is adequate. This is not true. For proper randomization, all possible assignments of treatments to experimental units must be equally likely to appear. An alternative but fully equivalent requirement, which is often easier to verify, is that each pair of treatments (control can be thought of as a treatment) has exactly the same chance of being assigned to every pair of experimental units (Hader, 1973).

Why randomize? Cox (1958, p. 85) states the positive advantages of randomization as assurances that:

(a) “In a large experiment it is very unlikely that the estimated treatment effects will be appreciably in error.”

(b) “The random error of the estimated treatment effects can be measured and their level of statistical significance examined, taking into account all possible forms of uncontrolled variation.”

3.2.3 Ethical Considerations

The ethical considerations are complicated and difficult in studies involving humans. In the clinical trials setting, and in fact in any experimental situation that deals with human subjects, the problem of whether to randomize treatment allocation tends to be confounded with the question of ethics. Is it ethical for a physician to allow some random device decide whether a patient is to receive a specific treatment?

3.2.4 Study Design

Steps to design an experiment include the design itself, calculating the sample size and performing randomisation. Important goals and purposes include:

1. To understand the objectives and constraints clearly, state what quantities are to be estimated, and identify what hypotheses are to be tested.

2. To consider possible designs that are tailored to the aims of the experiment and yet satisfy all ethical, physical, and financial constraints.
3. To evaluate the proposed design to establish that the objectives can be achieved, that the estimates are likely to have sufficient precision, and that any proposed tests of hypotheses have adequate power.

### 3.3 Study Design and Background of Study: Citrulline Bioavailability in Healthy Subjects

Despite many positive reports correlating fasting plasma citrulline concentrations to the degree of decreased enterocyte mass in various small intestinal disorders (Crenn et al., 2000; Crenn et al., 2003; Papadia et al., 2007; Papadia et al., 2010), Luo et al. (2007) reported no relation between fasting plasma citrulline concentrations and intestinal absorptive capacity in patients with short bowel syndrome. This latter finding was in line with data in patients with villous atrophy caused by coeliac disease and in patients with short bowel syndrome (Peters et al., 2007c; Peters et al., 2008b). Besides, Papadia et al. (2007) reported a quadratic (and not a linear) correlation between fasting plasma citrulline concentration and remnant small bowel length, indicating that decreased plasma citrulline concentrations predominantly reflect the extremes of disease spectrum.

Most of the circulating plasma citrulline is derived from glutamine conversion through the glutamate to ornithine pathway in the enterocyte (Curis et al., 2005). Other amino acids such as proline, glutamate and arginine may also serve as precursors for citrulline. Supply of glutamine to the enterocyte occurs from both the arterial blood as well as from the intestinal lumen at the rate of 25%-33%, and 66%, respectively. Van De Poll et al. (2007a); Van De Poll et al. (2007b) recently reported glutamine consumption by the intestine to be dependent on glutamine supply and observed that 13% of intravenously administered glutamine taken up by the intestine was converted into citrulline. Citrulline appears to be an important source of nitric oxide, increasing even more the availability of it through arginine (Osowska et al., 2008). 3-Methylhistidine is another important amino acid which has been tested as an absorption marker for the intestine and could function effectively as a way to detect a signal to noise ratio. As glutamine is the natural precursor of citrulline while arginine has been mentioned to produce citrulline, we hypothesized that loading the enterocyte with orally administered citrulline or glutamine or arginine or 3-methylhistidine might increase citrulline output to an extent reflecting enterocyte functional capacity. The rational for choosing each
amino acid will be described with details and references in section 3.3.2. Each amino acid will be given separately from the others in a cross-over design explained in section 3.3.4.

A similar experiment has been executed by Peters et al. (2007c); Peters et al. (2008b), commonly referred as *citrulline generation test*, but the authors did not include control challenges in their study, such as arginine, citrulline or 3-methylhistidine. This study did not include a comparison with placebo as well and open questions worth examining remained. Their results indicated that following the oral bolus of glutamine, plasma citrulline concentrations showed a time dependent rise in healthy subjects of 44% with higher rates of increase in healthy subjects compare to coeliac disease patients. Additionally, in patients with short bowel syndrome, the citrulline generation test indicated a higher incremental area under the curve till 90 minutes in adapted (defined as over 24 months since their final digestive circuit modification) compare to non-adapted cases with borderline significance but overall fasting plasma citrulline concentrations had poor test characteristics for detection of decreased intestinal energy absorption capacity in patients with enterocyte damage. Hence, their challenge test indicated an interesting potential but left many unanswered questions due to the fact that there weren’t control challenges, design biases with unbalanced groups and possible confounding bias (see Figure 2.2 from the previous chapter).

### 3.3.1 Hypotheses

My primary hypothesis is that a single oral bolus of glutamine or citrulline or arginine or 3-methylhistidine or placebo results in a time-dependent increase in plasma citrulline concentrations in healthy subjects in order to establish a normal range before repeating the doing the same procedure later on in intestinal failure patients. I hypothesize that this loading to reflect enterocyte mass, thus showing a delayed or attenuated citrulline response in patients with intestinal failure. 3-Methylhistidine and placebo will serve as controls allowing to test with rigour whether citrulline, arginine or glutamine have an effect.

Secondary Hypotheses:

1. Are there differences in the bioavailability of citrulline after giving the five treatments (3-methylhistidine, arginine, glutamine, citrulline or placebo)?
2. Is there a significant difference in the period which each is given? Each amino acid will be given in a difference sequence and this could produce a period bias. This is discussed below in study design.

3. Is there a significant difference between the sequences of treatment?

4. I will investigate as well the bioavailability of the amino acids glutamine, arginine, ornithine and 3-methyl-histidine. These are related to the boluses given because they are connected through the biochemical pathways discussed in Chapter 1. For these amino acids, I postulate as well the same questions:
   a. Are there differences in the bioavailability of these amino acids after giving the five treatments (3-methylhistidine, arginine, glutamine, citrulline or placebo)?
   b. Is there a significant difference in the period which each is given for these amino acids?
   c. Is there a significant difference between the sequences of treatment for these amino acids?

### 3.3.2 Rationale for Using these Particular Amino Acids

Citrulline has shown a high diagnostic sensitivity in previous studies with intestinal clinical conditions, as discussed in Chapter 2. Arginine and glutamine are precursors of citrulline. For 3-methylhistidine, I believe the following reasons make it important for using it as a challenge:

- The absorption of the free 3-methylhistidine is rapid.
- The serum levels are proportional to the amount of ingested muscular proteins; thus, it may be a useful tool for studying the pathophysiology of protein absorption in man.
- It is intended to be used as a marker that will standardize the other measurements, giving greater discrimination (tolerance test).

### 3.3.3 Pharmacokinetics

Arginine and the related amino acids glutamine and citrulline are widely used at pharmacological dosages for various purposes in a number of situations, e.g., athletes, bodybuilders, elderly and immunocompromised patients. When studied in
healthy subjects, the main aim of amino acid supplementation is to elicit growth hormone secretion or to sustain nitric oxide production.

**Arginine.** In a study by Tangphao et al. (1999), 10 healthy volunteers (6 males, 4 females) aged 23-52 years received 10 g of L-arginine in 100 mL water. Fürst (2001) mentions that we can reach even to 20 g of oral amino acid; hence, according to this data, arginine was administered orally in 20 g for one single dose, followed by a washout period of one week.

**Glutamine.** L-glutamine is a protein amino acid found in proteins of all life forms. It is classified as a semi-essential or conditionally essential amino acid. This means that under normal circumstances the body can synthesize sufficient L-glutamine to meet physiological demands. However, there are conditions where the body cannot do so. Recently, L-glutamine has come to be regarded as one of the most important amino acids when the body is subjected to such metabolic stress situations as trauma (including surgical trauma), cancer, sepsis and burns. Under such conditions, L-glutamine becomes an essential amino acid, and it is therefore very important to ensure adequate intakes of the amino acid in order to meet the increased physiological demands created by these situations.

L-glutamine is the most abundant amino acid in the body, and plasma glutamine levels are the highest of any amino acid. L-glutamine is predominantly synthesized and stored in skeletal muscle. The amino acid L-glutamate is metabolized to L-glutamine in a reaction catalysed by the enzyme glutamine synthase. The typical dietary intake of L-glutamine is 5 to 10 g daily. The dose can reach to 20 g (Fürst, 2001), and Ziegler et al. (1990) reached the dose to 0.3 g/kg (around 21 g in a 70 kg person). In the present study, L-glutamine was administered orally in one single dose of 20g, followed by a washout period of one week.

**Citrulline.** Citrulline is a non-protein amino acid that is present in substantial amounts in watermelon (*Citrullus vulgaris*), with a mean content of 2.1 mg/g fresh weight, ranging from 0.5 to 3.6 mg/g according to variety (Curis et al., 2005). A pharmacokinetic study was performed in six healthy adults receiving 3.3 kg wet weight of the red fruit of a ripe watermelon (Mandel et al., 2005); the study is limited by uncertainty as to the actual amount of citrulline ingested, but the average citrulline intake was probably around 7 g. Moinard et al. (2008) studied eight young male healthy adults (age: 27.6 ± 1.5 years; BMI = 22.3 ± 0.5 kg/m²) who received
2 g, 5 g, 10 g, or 15 g of citrulline in random order on separate occasions, with a 15 day washout between doses. The dose can reach to 20 g (Fürst, 2001), which was administered orally in the present study in one single dose, followed by a washout period of one week.

**3-Methylhistidine.** This amino acid is produced by methylation of the actin and myosin peptide chains in the muscle. Metabolism after intravenous administration of L-3-methylhistidine involves excretion in the urine of 75% of the administered dose in 24 hours and 95% in 48 hours. The plasma disappearance curves of [\textsuperscript{14}C]3-methylhistidine suggested a half-life of approximately 130 minutes (Long et al., 1975). Bachmann et al. (1984) used 3-methylhistidine for the assessment of intestinal protein absorption by studying its plasma kinetics for potential use as a marker of protein degradation. Oral 3-methylhistidine was given in doses 20 g, 60 g or 120 mg. The absorption of the free 3-methylhistidine is rapid and reaches a peak after 75 minutes. The peak is delayed after meat ingestion and reaches a maximum after 180 minutes. Peak height and area under the plasma concentration versus time curve were proportional to the dose received, indicating first order kinetics for 3-methylhistidine. It has a half-life of 12.2 hours. Hence, 3-methylhistidine was administered orally as 120 mg for one single dose, followed by a washout period of one week.

**Placebo.** Dextrose (glucose) was used in a dose of 20 g in this study. This is line with the study of Bachmann et al. (1984), who used glucose as placebo against 3-methylhistidine, and Figueroa et al. (2009), who used maltodextrine against oral citrulline.

NOW Foods (Bloomingdale, IL, R&D Scientist Dr L. Ber) kindly provided glutamine, citrulline, arginine and placebo for the study; 3-methyl-histidine was used from Sigma-Aldrich.

### 3.3.4 Trial Design

For the detection of difference between these four loadings and placebo, a crossover design was chosen. It is a randomised cross-over single-site study on the bioavailability of predominantly citrulline after an oral bolus of citrulline, glutamine, arginine, 3-methylhistidine or placebo in healthy subjects. Secondary measurements included measurements of the amino acids glutamine, arginine,
ornithine and 3-methylhistidine as well. In order to have a balanced design, free of carryover effects, each participant was allocated in a random manner to one of the ten sequences of the Williams balanced cross-over design, the optimum design for reducing carryover effect in crossover designs. After screening, the participants began receiving the first oral load of the sequence they were randomised to and subsequently had the appropriate blood samples taken. The first load and measurements lasted six hours. After this, the participant had a washout period of one week and returned for their second loading and measurements. This was repeated five times for each participant and then the study ended. The detailed diagram for each participant is shown in Figure 3.1. In case participants dropped out, the sequence was allocated again to a new participant.

3.3.4.1 Statistical Theory behind Crossover Designs

A crossover design is a modified, randomized block design in which each block receives more than one formulation of a drug at different periods.\(^1\) A block may be a subject or a group of subjects. Subjects in each block receive a different sequence of formulations. A crossover design is called a complete crossover design if each sequence contains each of the formulations (Chow and Liu, 2008). For a crossover design, it is not necessary that the number of formulations in each sequence be greater than or equal to the number of formulations to be compared. We shall refer to a crossover design as a \(g \times p\) crossover design if there are \(g\) sequences of formulations administered at \(p\) different periods. For bioavailability and bioequivalence studies, the crossover design is viewed favourably by the US Food and Drug Administration (FDA) and other regulatory agencies in the world, such as the European Medicines Agency (EMA), because of the following advantages:

1. Each subject serves as their own control. It allows a within-subject comparison between formulations.

2. It removes the inter-subject variability from the comparison between formulations.

\(^1\)A crossover design is an experimental design in which each experimental unit (subject) receives a series of experimental treatments over time. The order in which an experimental unit receives the treatments is called a sequence (example, ABC). The time interval during which a treatment is administered to a subject is called a period. A period could range from a few minutes to several months depending on the study. Sequences usually involve subjects receiving a different treatment in each successive period. However, one or more treatments may occur more than once in any sequence (example, AABBB). Treatments and periods are compared ‘within subject’, i.e. each subject acts as its own control. Therefore any component that is related to subject differences is removed from treatment and period comparisons. Carryover effects are residual effects from a previous treatment manifesting themselves during subsequent periods (Shanga, 2003).
3. With a proper randomisation of subjects to the sequence of formulation administrations, it provides the best unbiased estimates for the differences (or ratios) between formulations.

4. This design has the need of fewer subjects than a parallel design, with an important decrease on costs of the study.

The use of crossover designs for clinical trials has been extensively discussed in the literature. See, for example Senn (2002) and Jones and Kenward (2003).

**Washout and Carryover Effects**

It is helpful to introduce the concepts of washout and carryover effects (or residual effects) in a crossover design because the presence of carryover effects usually has an influence on statistical inference of bioavailability between formulations.

The washout period is defined as the rest period between two treatment periods for which the effect of one formulation administered at one treatment period does not carry over to the next. In a crossover design, the washout period should be long enough for the formulation effects to wear off so that there is no carryover effect from one treatment period to the next. The washout period depends on the nature of the drug. A suitable washout period should be long enough to return any relevant changes that influence bioavailability to baseline (usually, at least five times the blood-plasma elimination half-life of the active ingredient, therapeutic moiety or its metabolite, or the decay of the immediate pharmacological effect since the last sampling time point of the previous period).

If a drug has a long half-life or if the washout period between treatment periods is too short, the effect of the drug might persist after the end of dosing period. In this case, it is necessary to distinguish the difference between the direct drug effect and the carryover effects. The direct drug effect is the effect that a drug product has during the period in which the drug is administered, whereas the carryover effect is the drug effect that persists after the end of the dosing period. Carryover effects that last only one treatment period are called first-order carryover effects. A drug is said to have $c$-order carryover effects if the carryover effects last up to $c$ treatment periods. In bioavailability and bioequivalence studies, however, it is unlikely that a drug effect will carry over more than one treatment period because a sufficient length of washout is usually considered.
Crossover Designs for Three or More Formulations

The crossover designs for comparing three or more formulations are much more complicated than those for comparing two formulations. For comparing three formulations of a drug, there are a total of three possible pairwise comparisons between formulations: formulation 1 versus formulation 2, formulation 1 versus formulation 3, and formulation 2 versus formulation 3. It is desirable to estimate these pairwise differences in average bioavailability between formulations with the same degree of precision. In other words, it is desirable to have equal variances for each pairwise differences in average bioavailability between formulations (i.e. $V(\hat{F}_i - \hat{F}_j) = \nu \sigma_e^2$) where $\nu$ is a constant and $\sigma_e^2$ is the intra-subject variability.

Designs with this property are known as variance-balanced designs. It should be noted that, in practice, $n$ may vary from design to design. Thus, an ideal design is one with the smallest $n$, such that all pairwise differences between formulations can be estimated with the same and possibly best precision. However, to achieve this goal, the design must be balanced. A design is said to be balanced if it satisfies the following conditions (Jones and Kenward, 2003):

1. Each formulation occurs only once with each subject.
2. Each formulation occurs the same number of times in each period.
3. The number of subjects who receive formulation $i$ in some period followed by formulation $j$ in the next period is the same for all $i \neq j$.

Under the constraint of the number of periods ($p$) being equal to the number of formulations ($t$), balance can be achieved by using a complete set of orthogonal Latin squares (Jones and Kenward, 2003). However, if $p = t$, a complete set of orthogonal Latin squares consists of $t \times (t-1)$ sequences except for $t = 6$. As a result, when the number of formulations to be compared is large, more sequences and consequently more subjects are required. This, however, may not be of practical use.

A more practical design has been proposed by Williams (1949). This shall be referred to as a Williams design. A Williams design possesses balance property and requires fewer sequences and periods. The algorithm for constructing a Williams design with $t$ periods and $t$ formulations is summarized in the numerical steps mentioned in the next section (Jones and Kenward, 2003).
Williams Designs: Construction and Randomization

The Williams design is a special case of the cross-over and Latin square designs. A Latin square, in which every treatment is represented once, and once only, in each column and in each row, yields uniform cross-over designs; it represents uniformity both within periods and within sequences (Wang et al., 2009). To achieve the highest possible efficiency, the design must be balanced. In a balanced design, each of the treatments occurs the same number of times in each period and the number of subjects who receive treatment $i$ in one period and treatment $j$ in the next period is the same for all $i \neq j$. Fisher and Yates (1963) give examples of Latin square designs. Some of them have additional properties that other Latin squares do not. For example, in the design listed in Table 3.1, every treatment follows every other treatment the same number of times. Such a cross-over design, which was introduced by Williams (1949), is said to be balanced with respect to first-order carry-over effects. Williams’s designs require fewer subjects than those based on complete sets of orthogonal Latin squares, which is another way to produce balanced cross-over designs. If the number of treatments to be tested is even, the design needs only one Latin square, called the Williams single; otherwise it consists of two Latin squares (except in a few special cases), called the Williams pair (Bate and Jones, 2006).

Practical Construction of a Williams Design

Although Williams (1949) describes the steps needed to construct one of his designs, a more easily remembered algorithm is given by Sheehe and Bross (1961). The details of the construction methods of a Williams design are found in Jones and Kenward (2003). An encyclopaedia review article on the related subject is found in Bate and Jones (2006).

A Williams design can be generated by re-arranging a ‘standard’ Latin square design, in which the first row and column consists of the letters written in alphabetical order. The practical steps in Sheehe and Bross (1961) algorithm are as follows Jones and Kenward (2003):

1. Number the treatments from 1 to $t$ ($t =$ the number of treatments to compare).
2. Start with a cyclic $t \times t$ Latin square. In this square the treatments in the $i$th row are $i, i + 1, \ldots, t, 1, 2, \ldots i-1$. 
3. Interlace each row of the cyclic Latin square with its own mirror image (i.e. its reverse order). For example, if $t = 4$, the first row of the cyclic square is 1, 2, 3, 4. Its mirror image is 4, 3, 2, 1. When the two sequences are interlaced, the result is 1, 4, 2, 3, 3, 2, 4, 1.

4. Slice the resulting $t \times 2t$ array down the middle to yield two $t \times t$ arrays. The columns of each $t \times t$ array correspond to the periods, the rows are the treatment sequences, and the numbers within the square are the treatments.

5. If the number of treatments is even, any one of the two $t \times t$ arrays are chosen. If the number of treatments is odd, both arrays are used (e.g. for $t = 5$, both Latin squares are used producing 10 sequences).

The design for $t = 4$ obtained by using this algorithm and choosing the left-hand square is shown in Table 3.1.

The Statistical Model of a Crossover Design (Matthews, 1988)

In a crossover design, because the direct drug effect may be confounded with any carryover effects, it is important to remove the carryover effects from the comparison if possible. To account for these effects, the following statistical model is usually considered. Let $Y_{ijk}$ be the response (e.g., area under the curve) of the $i$th subject in the $k$th sequence at the $j$th period. Then:

$$Y_{ijk} = \mu + S_{ik} + P_j + F_{(j,k)} + C_{(j-1,k)} + \varepsilon_{ijk}$$

where

- $\mu$ is the overall mean
- $S_{ik}$ is the random effect of the $i$th subject in the $k$th sequence, where $i = 1, 2, \ldots, g$
- $P_j$ is the fixed effect of the $j$th period, where $j = 1, \ldots, p$ and $\sum P_j = 0$.
- $F_{(j,k)}$ is the direct fixed effect of the formulation in the $k$th sequence which is administered at the $j$th period, and $\sum F_{(j,k)} = 0$.
- $C_{(j-1,k)}$ is the fixed first-order carryover effect of the formulation in the $k$th sequence which is administered at the $(j-1)$th period, where $C_{(0,k)} = 0$; and $\sum C_{(j-1,k)} = 0$. 
• \( \varepsilon_{ijk} \) is the (within-subject) random error in observing \( Y_{ijk} \).

It is assumed that \( S_{ik} \) are independently and identically distributed with mean 0 and variance \( \sigma_S^2 \), and \( \varepsilon_{ijk} \) are independently distributed with mean 0 and variance \( \sigma^2 \), where \( t = 1, 2, \ldots, L \) (the number of formulations to be compared); and \( \varepsilon_{ijk} \) are assumed mutually independent. The estimate of \( \sigma^2_t \) is usually used to explain the inter-subject variability, and the estimates of \( \sigma^2_t \) are used to assess the intra-subject variabilities for the \( t \)th formulation (Chow and Liu, 2008).

A term for the carryover treatment effect is usually introduced into the model to allow for the absence or inadequacy of washout periods. If the effect of treatment does persist into the period following the period of administration, but a carryover term is not included in the model, then the estimates of the direct treatment effect will be biased. For a more detailed discussion of the issues involved, see Abeyasekera and Curnow (1984). If there is a carryover effect of the treatment, then it must be decided whether it is the direct or carryover effect that is of interest. The relevance of the ‘total effect’ \( F_{(j,k)} + C_{(j-1,k)} \) and the possibility of modelling carryover effect using fewer parameters, perhaps by assuming proportionality between direct and carryover effects, could be considered (Patterson and Lucas, 1962).

The aim is to devise designs that allow the treatment effect in the equation to be estimated within-unit and within-period. If the \( S_{ik} \) term is taken to be fixed, then any estimator of treatment effect must be within-unit. Some writers assume that the unit effects are random: this means that one can also use between-unit information in one’s analysis. For quantities that can be estimated within-unit, it is seldom profitable to use this less precise source of information.

Between-unit information is sometimes used when a treatment \( \times \) period interaction is being considered. Good discussions of the problems of treatment \( \times \) period interactions in the context of the simple two-treatment, two-period crossover design are given by Hills and Armitage (1979) and Cox and Reid (2000). Little has been written of such interactions in more complicated designs. In particular the relationship between treatment \( \times \) period interactions and the set of carryover effects, of all orders, terms having the same number of degrees of freedom, does not seem to have been investigated.
Consideration of unit $\times$ treatment interaction does not seem to have received any attention in the literature. Such a term could be of interest; in a medical context it may be of importance to know which patients react favourably to the treatments. If there is a measurable feature of the units that could influence treatment effects, then it may be instructive to consider the interaction between this quantity and treatment effects. However, if one could not characterise the patients through such a measurement, then consideration of unit $\times$ treatment interaction might be less valuable.

A final remark on the model concerns the issue of baseline measurements: these are measurements taken on the response variable (or, occasionally, on some closely related variable) at the start of the experiment, and perhaps also at the start of each treatment period. Baseline measurements taken only at the beginning of the experiment are of no relevance to within-unit comparisons. It is less well understood how baseline readings taken at the start of each treatment period, perhaps after a washout period, should be used. Kershner and Federer (1981), Laska et al. (1983) and Laska and Meisner (1985) assume that the baseline readings follow model but with the direct treatment effect omitted, and with an error term that is independent of that for the response variables. These authors use this approach in their comparisons of various two-treatment designs. Identifying the parameters in the models for the baseline and response variables in this way is open to a number of practical criticisms.

Many of the present design issues will be discussed with respect to present design in further sections of the present chapter.

### 3.3.5 Selection of Subjects

#### 3.3.5.1 Inclusion Criteria

1. Healthy volunteers fasted over the previous night.
2. Age equal or over 18.
3. No significant past illness, subject to investigator’s judgement.
4. All volunteers provided written informed consent before study initiation.
3.3.5.2 Exclusion Criteria

1. Having drunk coffee or alcohol during the previous night.

2. Person on regular medication or with a significant past medical illness (e.g. cardiovascular, hepatic, renal, psychiatric, neurologic, hematologic, or metabolic disease; drug or alcohol abuse within 2 years before the start of the study; smoking; HIV, hepatitis B virus, or hepatitis C virus infection)

3. Consumption of any prescribed or over-the-counter drug within 2 weeks before the start of the study;

4. Participation in a similar study within the past 6 months.

5. Female subjects were not pregnant, planning to become pregnant, or breastfeeding at the time of the study.

3.3.6 Study Procedures and Schedule of Assessments

3.3.6.1 Randomisation Procedures

Each participant was randomized to one of the 10 sequences of the Williams design discussed above. This randomisation list was produced with a SAS algorithm which is discussed in the statistical section of this protocol.

3.3.6.2 Screening Assessments

Screening assessments involved determining whether potential participants fulfil the inclusion and exclusion criteria. This was done:

1. with a thorough medical history of all previous diseases and symptoms that might or might not be affecting the participant. The participant was asked if he/she had drunk coffee or alcohol during the previous night.

2. with a detailed history of regular medication.

3. through a physical examination of approximately 10 minutes on all systems of the body (gastrointestinal, cardiovascular, respiratory, general appearance).

4. with a urine pregnancy test.
3.3.6.3 Schedule of Evaluations and Procedures

All clinical study evaluations prior to screening, during screening and after screening were performed according to the schedule shown in Table 3.2. Visits were scheduled at as consistent a time as possible that were routinely available for the subject and investigator, preferably in the morning. There was a visit window from Visit 1 (V1) to Visit 5 (V5) of ± 1 day. All visits were calculated with references to V1, not with reference to the previous visit. When 20 subjects had completed V5 no further screening was performed.

This randomised-sequence, open-label, 5-period crossover study was conducted from August 2011 to May 2012. After an overnight fast, subjects received a single oral dose of either one of the amino acids or placebo with 200 mL of water. There was a 7-day washout between periods, after which subjects received the other formulation. Randomization was performed using a random number table and the treatment sequences were balanced with a Williams design. Written informed consent was obtained from all subjects after the nature and purpose of the study had been explained and before clinical screening, which included physical examination and laboratory tests. After signing informed consent, patients underwent clinical examination. Laboratory analyses consisted of determination of blood haemoglobin levels, haematocrit, and total white blood cell counts. Biochemistry and haematology safety were performed only at screening.

Subjects were confined to the centre during each visit for the assessments and blood sampling (6 hours). They received a payment for travel expenses regardless of whether they completed the study. To avoid repeated venepuncture, a peripheral venous catheter was placed during each visit in an antecubital vein and flushed with 0.5 mL of a 1:20 dilution of sodium heparin in saline after each sampling. Before collection of the next sample, 5 mL of blood was discarded to ensure that the sample was free of the flushing solution. The peripheral venous catheter was removed at the end of each visit and a new one was inserted at the beginning of the next visit.

The study protocol and informed consent forms were approved by the East London Research Ethics Committee (Ref. No 11/H0703/4) and the R&D Department of UCL/UCLH (Ref. No 10/0458). The study was performed in accordance with the Declaration of Helsinki (revised, Tokyo 2004) (World-Medical-Association, 2013) and the Good Clinical Practice guidelines (EMEA, 1997).
3.3.7 Laboratory Procedures

3.3.7.1 Plasma Amino Acid Measurement Method

For determination of plasma amino acid levels (citrulline, arginine, glutamine, ornithine, 3-methyl-histidine), blood samples (8 mL) were collected in potassium EDTA with 0.1 mL 1 sodium metabisulfite solution at the following times: predose and 15, 30, 45, 60, 90, 120, 180, and 240 minutes. Samples were centrifuged immediately at approximately 3000 g for 10 minutes at 4°C. The resulting plasma was separated, and aliquots were stored at -80°C until required for analysis. As stated by (Neveux et al., 2003), the deproteinated samples can be stored at -70°C until analysis with no loss of amino acids, including glutamine, for at least several months to a year. Amino acids were separated and quantified by mass spectrometry. All samples were analysed with mass spectrometry at UCL Cancer Centre under the supervision of Dr Cali Hyde. The kit EZ:Faast was used which included internal standards and solvents for the study. Product information: http://www.phenomenex.com/Products/AminoAcidDetail/EZfaast.

The EZ:faast amino acid analysis procedure consists of a solid phase extraction step followed by a derivatization and a liquid/liquid extraction; derivatized samples are quickly analysed by liquid chromatography-mass spectrometry. The solid phase extraction is performed via a sorbent packed tip that binds amino acids while allowing interfering compounds to flow through. Amino acids on sorbent are then extruded into the sample vial and quickly derivatized with reagent at room temperature in aqueous solution. Derivatized amino acids concomitantly migrate to the organic layer for additional separation from interfering compounds. Organic layer is then removed, evaporated, and re-dissolved in aqueous mobile phase and analysed on a LC/MS system. Total sample preparation time takes around 8 minutes and analysis is performed in around 12 minutes for a total start to finish time of around 20 minutes.

3.3.7.2 Summary of Procedure

1. For each sample line up one glass sample preparation vial in the vial rack.
2. Pipette 100 µL sample and 100 µL Reagent 1 into each sample preparation vial.
3. Attach a sorbent tip to a 1.5 mL syringe; pass the solution in the sample preparation vial through the sorbent tip by slowly pulling back the syringe piston.

4. Pipette 200 µL Reagent 2 (Washing Solution) into the sample preparation vial.

5. Slowly pass the solution through the sorbent tip and into the syringe barrel.

6. Detach the sorbent tip, and discard the liquid accumulated in the syringe.

7. Pipette 200 µL Eluting Medium (prepared fresh each day) into the sample preparation vial.

8. Pull back the piston of a 0.6 mL syringe halfway up the barrel and attach the sorbent tip.

9. Wet the sorbent with Eluting Medium; stop when the liquid reaches the filter plug in the sorbent tip.

10. Eject the liquid and sorbent out of the tip and into the sample preparation vial. Repeat, until all sorbent particles in the tip are expelled into the sample preparation vial. Discard the empty tip.


12. Emulsify by repeatedly vortexing the solution for about 5 seconds. Allow reaction to proceed for about 1 minute.

13. Vortex the solution again for a few seconds to re-emulsify the content of the vial. Allow the reaction to proceed for at least one additional minute.

14. Using the Microdispenser, transfer 100 µL Reagent 5, and re-emulsify by vortexing for about 5 seconds. Let the reaction proceed for 1 minute.

15. Transfer part of the (upper) organic layer (50-100 µL) with a Pasteur pipette into an autosampler vial. Avoid transferring aqueous layer along with the organic layer. Evaporate the solvent slowly to dry under a gentle stream of nitrogen (maximum 10 minutes). Re-dissolve amino acid derivatives in 100 µL (or less) of a mixture of LC mobile phase components A:B 1:2 (v/v). Transfer the reconstituted sample into an insert, and place the insert in the same autosampler vial. The reconstituted sample is ready for LC/MS analysis.
3.3.7.3 LC-MS Analysis

**LC Settings**

Mobile phase:
- A: 10 mM Ammonium formate in water
- B: 10 mM Ammonium formate in methanol

Gradient: 0.00 min 68%B 13.00 83%B 13.01 68%B 17.00 68%B

Re-equilibrate column for 4-6 min before next injection depending on HPLC system used.

Flow rate: 0.50 mL/min. for 3.0 mm ID column 0.25 mL/min. for 2.0 mm ID column

Column temperature: 35 °C

Injection volume: 1 µL

**MS Settings**

Either ESI or APCI was used

Mode: Positive Ion

Scan range: 100-650 m/z

ESI ion source temperature: 365 °C (Bruker); 425 °C (AB API3000)

APCI ionization chamber temperature: 450 °C

3.3.8 Statistical Considerations

3.3.8.1 Primary Endpoints

The primary end point for this study was the Area under the Curve (AUC) for citrulline after each oral load. This is the fraction of the administered dose that reaches the systemic circulation. Bioavailability is 100% for intravenous injection. It varies for other routes depending on incomplete absorption, first pass hepatic metabolism etc. Thus one plots plasma concentration against time, and the bioavailability is the AUC.

3.3.8.2 Secondary Endpoints

- AUC of all other amino acids after oral load.
- Peak concentration ($C_{max}$, µmol/L) of all amino acids after oral load.
- Increment (%) of all amino acids after oral bolus.
• Time to peak ($T_{max}$, min) of all amino acids after oral load.

• Time of maximum concentration (TOMC)

• Slope of line between Concentration at Baseline and $C_{max}$ ($\mu$mol/L/min) for all amino acids.

### 3.3.8.3 Sample Size Calculation

Since this was an exploratory study with no similar in the literature, there were no sources for prior variability to allow subsequent sample size computation. However, the design of a balanced crossover study created a few conditions which allowed for sample size estimation. Thus, the restraint of the Williams design was that the number of subjects must be a multiple of 10. Thus, our chosen sample size is 20, in order to avoid possible underpowered study on 10 healthy subjects (this was reviewed by the Ethics Committee which agreed that a sample size of 20 participants was likely an appropriately powered study whilst 10 participants would be an underpowered study). Approximately 2 patients were recruited per week, based on the advertisements placed.

#### Theory for Sample Size Calculation

The sample is the group of subjects on which the study is performed. The choice of the sample requires qualitative and quantitative considerations. Among the qualitative aspects of the sample selection, crucial is the need to ensure that the sample is representative of the population to which one wants to extend the conclusions of the study. Among the quantitative ones, crucial is the need to quantify the concept of 'sufficiently large sample', i.e. of a sample large enough to allow the detection of the treatment effect, separating it from the variability of the phenomenon, with an acceptable degree of certainty. This is achieved through statistical methods. The size of the sample required for a given study depends on the magnitude of the signal, the risks we are willing to accept of making type I and type II errors, the type and variability of the end-point(s), the design of the study, and the number of treatments and primary end-points. Once the information on the size of the sample required for a given study is obtained, it is essential to evaluate the feasibility of the study, based on practical considerations, such as the number of patients we are likely to obtain from the participating centres, the study duration
considered acceptable and the projected costs. If the study is not feasible, sometimes changes in the design will allow the researcher to conform to the limits set by the abovementioned practical considerations. However, in other cases, it is best to give up. This is much less frustrating than proceeding with a study which is bound to fail (Bacchieri and Della Cioppa, 2007). In exploratory studies it might prove the case to base sample size on statistical estimations that will likely provide the necessary power for the study.

3.3.8.4 Statistical Analysis Plan

Primary Endpoint Analysis

The AUC of citrulline for the time 0-6 hours (AUC$_{0-6}$) was calculated by the trapezoidal rule. The AUC from the last experimental time to infinity (AUC$_{6-\infty}$) was calculated by extrapolation, dividing the last measured plasma concentration value by the apparent elimination rate constant ($k_e$). The AUC$_{0-\infty}$ was calculated by adding AUC$_{0-6}$ and AUC$_{6-\infty}$. All AUCs corrected for baseline concentration, which were taken as the concentration at $t = 0$ hours, are termed $\Delta$AUC.

Three factors were considered for the analysis: treatment, patient and period of administration. The experiment followed a double Latin square design assuming an additive model and no interaction between the two squares. Three-factor ANOVA followed by Tukey’s honestly significant differences post hoc test was used to estimate effects of each of the three factors on all the above computed plasma pharmacokinetic parameters. Tests were applied to the natural logarithm-transformed values of $\Delta$AUC in order to homogenise variances, if their distribution was found to be non-parametric.

Secondary Endpoint Analysis

AUC$_{0-6}$ for all other amino acids were smoothed by cubic spline interpolation and the interpolated curve was derived numerically in order to obtain a smoothed $C(t)$ curve. $C_{max}$ and $T_{max}$ were deduced from this smoothed $C(t)$ curve. All $C_{max}$ corrected for baseline concentration were termed $\Delta C_{max}$.

Clearance (Cl) was evaluated as $Cl = \text{dose}/\Delta \text{AUC}_{0-\infty}$. Apparent distribution volume ($V_d$) was computed as $Cl/k_e$. The apparent half-life of elimination ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/k_e$. 
Pharmaceutical and Statistical Analyses

Amino acid plasma concentrations were analysed as a function of time. The following pharmacokinetic parameters were obtained for each formulation: $C_{max}$, Time of maximum concentration (TOMC), $AUC_{0-t}$ (where $t$ is the last time point with a measurable concentration), and $AUC_{0-\infty}$. $C_{max}$ and TOMC were obtained directly from the original data set, and $AUC_{0-t}$ was calculated using the linear trapezoidal rule. $AUC_{0-\infty}$ was calculated as $AUC_{0-t} + C_t/k_e$, where $C_t$ was the last measured concentration and $k_e$, the elimination rate constant, was calculated using linear regression of the log-linear portion of the plasma concentration–time curve. The half-life was calculated as $\ln(2)/k_e$. Mean values for $C_{max}$, $AUC_{0-t}$, and $AUC_{0-\infty}$ were adjusted for gender and body mass index using a multivariate analysis-of-variance model. Comparisons were performed with and without adjustment.

Applying a noncompartmental model to the concentrations of all amino acids, the pharmacokinetic parameters ($C_{max}$ and $AUC_{0-t}$) were compared by analysis of variance for a crossover design, taking into account the effects of formulation, period, sequence, and subject (Chow and Liu, 2008).

The Schuirmann and Anderson-Hauck tests were used to examine bioequivalence. The ratios and 90% CIs of $C_{max}$ and $AUC_{0-t}$ were calculated for both formulations, and 2 one-sided $t$-tests were employed to evaluate whether the 90% CIs of the geometric mean ratios (test:reference) for these parameters met the criteria for bioequivalence (i.e. if they were within the range from 80% to 120%) (Schuirmann, 1987; Anderson and Hauck, 2007; Chow and Liu, 2008).

Unless otherwise stated, data are presented as the mean (SD). Differences were considered significant at $p < 0.05$. Stata 12.0 and SPSS 22.0 were used for analyses. Missing values were replaced by the mean values of their categories.

**Randomisation**

To generate a proper Williams design, based the Sheehe-Bross algorithm, a simple Latin square is constructed at random, and then re-arranged (Sheehe and Bross, 1961). In a crossover trial, subjects are not randomized to treatment in the same sense as they are in a parallel-group design. In cross-over studies, only the treatment sequences are randomized (Jones and Kenward, 2003). Consequently, after the Williams design is constructed at random, the appropriate sequences are then randomly assigned to the subjects. In addition, equal numbers of subjects may be
allocated to all the sequences to ensure balance. Thus, the number of subjects required in the trial is usually a multiple of the number of sequences. In practice, there are usually several patients assigned to each sequence. The appropriate statistical software to design a Williams design study is the statistical package SAS. Hence, the SAS code for a Williams design and randomization for a $5 \times 5$ crossover trial and sample size = 20 was (Wang et al., 2009):

```
&WILLIAMS(
    TmtsNum=5,
    TmtNames= Cit Arg Gln 3-MH Placebo,
    Samplesize=20,
    OutRtf=D:\WilliamsN24.rtf,
    SeedNum=);
```

Below is a balanced set of sequences for five treatments. In this case 10 sequences are needed to keep the balance (and each treatment is followed by every other treatment twice), so multiples of 10 subjects are needed in the study:

1: A B C D E  
2: B D A E C  
3: D E B C A  
4: E C D A B  
5: C A E B D  
6: E D C B A  
7: C E A D B  
8: A C B E D  
9: B A D C E  
10: D B E A C

**Williams Design and Randomisation List for the Current Study**

This was produced with SAS code (SAS 9.2) and is shown in Table 3.3. Table 3.4 shows each sequence per subject.

**Outliers’ Analysis**

Outliers were analysed to investigate for influential or outlier observations. Heteroscedasticity was tested with the Breusch-Pagan / Cook-Weisberg test (Breusch and Pagan, 1979; Cook and Weisberg, 1983) and influential observations were identified per treatment with Cook’s distance (Cook, 1977, 1979) while DFBETAs were graphed per subject per treatment (Dehon et al., 2009).
A collection of variables is heteroscedastic if there are sub-populations that have different variabilities from others. The Breusch-Pagan / Cook-Weisberg test tests whether the estimated variance of the residuals from a regression or ANOVA are dependent on the values of the independent variables. In that case, heteroscedasticity is present. ANOVA is quite frequently robust, in a manner that results are resistant to departure from usual pre-requisites for performing ANOVA; nevertheless, influential points provide insight into the presence and sources of variability.

Cook’s distance (Cook’s D) is a commonly used estimate of the influence of a data point when performing regression analysis and ANOVA. There are different opinions regarding what cut-off values to use for spotting highly influential points: a simple guideline of $D > 1$ has been suggested while others have suggested $D > 4/n$, where $n$ is the number of observations; the latter is used in the present study. DFBETAs are another metric used to estimate and visualize influential observations. The DFBETA for a particular observation is the difference between the regression coefficient for an included variable calculated for all of the data and the regression coefficient calculated with the observation deleted, scaled by the standard error calculated with the observation deleted. The cut-off value for DFBETAs is $2/\sqrt{n}$, where again $n$ is the number of observations (Dehon et al., 2009).

### 3.4 Results

#### 3.4.1 Patients

Twenty Subjects were enrolled: 4 females and 16 males, mean age 22.3 years (SD = 4.7), mean height 1.75 m (SD = 0.06), mean weight 74.8 kg (SD = 11.9) and mean BMI 24.3 kg/m$^2$ (SD = 3.5).

#### 3.4.2 Method Validation

Extraction recovery of amino acids measured in plasma samples was adequate. Means, standard deviations and other statistical measures for retention time, area height, concentration ($\mu$mol/L), and internal standard (ISTD) area are shown in Table 3.5 and Table C.1, Appendix C. Dates of analysis are shown in Table C.2 and correspond to the different standard curves used for computing the concentration of plasma amino acids. The retention times for arginine, citrulline, glutamine,
ornithine and 3-methylhistidine were 3.06, 3.64, 3.73, 6.56, and 4.49 minutes, respectively. For each internal standard, retention times were similar. Linearity was achieved over a concentration range of 50 μmol/L to 400 μmol/L (Figures C.1-C.5, Appendix C), with the typical equation for the calibration curve reaching $R^2$ over 0.968, with most of them being approximately 0.999.

3.4.3 Bioavailability Analysis

3.4.3.1 Spaghetti Plots, Individual Subject Plots and Means Plots

Spaghetti plots, individual subject plots and means plots are first presented for each amino acid concentration (citrulline, arginine, glutamine, ornithine and 3-methylhistidine) and per treatment (citrulline, arginine, glutamine, placebo and 3-methylhistidine) (Figures 3.2-3.26). Time-dependent increases in citrulline, arginine, glutamine and ornithine levels post-ingestion of arginine, citrulline or glutamine are observed. Placebo and 3-methylhistidine ingestion do not seem to produce an effect. No inferences can be made though; this will be performed with statistical testing in the next section.

3.4.3.2 Crossover ANOVA for Means of Concentrations before Transforming Into Area under the Curve

Initially, an analysis of variance (ANOVA) for a crossover study with respect to each amino acid concentration under each treatment was performed. This analysis does not take into account the different time points, but it will generate initial proxy results when the area under the concentration-time curve (AUC) ANOVA is performed, which is the main result from a crossover study. The results are shown in the Table C.3 and in summary they are:

1. All amino acids appear to have been affected by the treatments significantly (ANOVA $p < 0.001$).
2. There isn’t a carryover effect with respect to citrulline concentrations but there is one with respect to arginine, glutamine, ornithine and 3-methylhistidine (ANOVA $p < 0.001$).
3. These results are only proxies to the final results when analysis is done with an ANOVA for the AUC. They are to be treated with caution but if similar patterns are observed with next results, this strengthens the results.
3.4.3.3 AUC Analysis

Means of AUC, \(C_{\text{max}}\) and TOMC

This information is shown in Table 3.6 and in Figure 3.27. This table is quite important because it depicts means and standard deviations (SDs) for the AUC, \(C_{\text{max}}\) and TOMC for each plasma amino acid measured according to treatment. The results reveal the following:

1. When the subject is loaded with citrulline there are increases in subsequent plasma levels of glutamine, citrulline and arginine (as compared with placebo) (greater AUC) (Table 3.6, Figure 3.27). When loaded with glutamine or arginine there are also increases in subsequent levels of glutamine, citrulline and arginine. The magnitude of these increases is greatest when the loading dose was of citrulline.

2. The effect of citrulline comes earlier in time compared to glutamine (greater TOMC).

3. There is a spread of effects – mostly with citrulline and least with glutamine – for the doses used in these experiments. Citrulline, arginine, glutamine and ornithine levels appear to be affected more when given citrulline and arginine than any other treatment.

4. The distribution of AUC and \(C_{\text{max}}\) is not normal as shown by the histograms (Figures C.6 and C.7) and Shapiro Wilk test \((p < 0.05)\).

Statistical inference testing of these figures is performed next to investigate any potential significant differences.

Crossover ANOVA for AUC and \(C_{\text{max}}\)

The AUC for each amino acid concentration didn’t follow a normal distribution (Shapiro Wilk’s test \(p\) value < 0.05 for all AUCs; see histograms Figures C.6 and C.7). Initial Kruskal-Wallis testing for non-parametric data revealed similar \(p\)-values with ANOVA, because ANOVA is a robust technique which is able to perform satisfactorily even when data are not parametric (Schmider et al., 2010). The results are shown in Tables 3.7 to 3.9, Table C.4 (Appendix C) and Figures C.8-C.40 (Appendix C). Overall:

1. There was no effect of age, gender and BMI on the AUC, \(C_{\text{max}}\) and TOMC (Figures C.8-C.22 for age, Figures C.23-C.37 for BMI and Figures C.38-C.40 for gender and MANOVA Table C.4; \(p < 0.05\)). This essentially indicates that
basic anthropometric characteristics and age have no effect on pharmacological post-ingestion measures.

2. Citrulline, arginine and ornithine concentrations were more affected by the treatment overall (crossover ANOVA $p < 0.05$, Tables 3.7 and 3.8) in comparison with glutamine and 3-methylhistidine which were not affected ($p > 0.05$). Both the AUC and the $C_{max}$ were affected indicating that overall post-absorptive concentrations and the maximum concentration were influenced by the type of treatment.

3. There was no carryover and period effect for all treatments, except for 3-methylhistidine which had a significant period effect (possibly due to small concentrations, $p < 0.05$; Tables 3.7 and 3.8). This confirms that the study design maintained low likelihood for carryover or period effects.

4. Citrulline administration increased arginine concentrations significantly compared to placebo and 3-methylhistidine. Citrulline loading also had a stronger effect than glutamine but not arginine ($p < 0.05$ from Tukey multiple comparisons Table 3.9; see also Table 3.6, Figure 3.27).

5. Arginine administration increased arginine concentrations significantly compared to placebo and 3-methylhistidine ($p < 0.05$ from Tukey multiple comparisons, Table 3.9). Additionally, arginine loading increased arginine concentrations with a stronger effect than glutamine but not compared to citrulline (although the increase was higher after arginine loading than after citrulline loading) (Table 3.9; see also Table 3.6, Figure 3.27).

6. Citrulline administration increased citrulline concentrations significantly compared to placebo and 3-methylhistidine (controls) as well as arginine and glutamine ($p < 0.05$ from Tukey multiple comparisons, Table 3.9). Increase of citrulline concentrations was larger with arginine loading compare to glutamine loading ($p < 0.05$ from Tukey multiple comparisons, Table 3.9).

7. Citrulline administration increased ornithine levels significantly compared to placebo and 3-methylhistidine (controls) as well as arginine and glutamine ($p < 0.05$ from Tukey multiple comparisons, Table 3.9).

8. Arginine administration increased ornithine levels compared to placebo and 3-methylhistidine (controls) as well as glutamine ($p < 0.05$ from Tukey multiple comparisons, Table 3.9); but less prominently than citrulline loading ($p < 0.05$ from Tukey multiple comparisons, Table 3.9).
3.4.3.4 Bioequivalence Testing

The bioequivalence of formulations was examined and results are presented in Table 3.10. Interestingly no treatment was equivalent to treatment with citrulline for producing equivalent post-absorptive concentrations \((p > 0.05)\). With respect to all other amino acid concentrations, equivalence was reached with all treatments (Anderson-Hauck test \(p < 0.05\)). Hence, it is noted that an effect has not been demonstrated but the absence of an effect cannot be inferred from this calculation.

3.4.3.5 Analysis of Outliers

There was heteroscedasticity in the plasma levels of citrulline, arginine, placebo and 3-methylhistidine but not glutamine (as measured by AUC) (Table 3.11). The percentage of influential observations in each plasma group was between 6% and 12%. Figure 3.28 is a graph of the DFBETAs per plasma amino acid levels (calculated in AUC) per treatment. Increased variability and more influential points were noted in the treatment groups for the plasma amino acid measured. For example, with respect to plasma citrulline AUC, citrulline treatment group had more influential points compare to the others. Variability in the placebo group was markedly less compared to the other treatments. This was similarly observed in the other sub-graphs of Figure 3.28. The placebo treatment group had increased variability with respect to measurement of plasma glutamine AUC, almost comparably to glutamine and arginine, indicating a need for caution when interpreting the ANOVA results for plasma glutamine AUC. Overall, although there are influential points in the placebo treatment groups across all plasma amino acids measured, their influential potential is much less prominent and less variable compared to the other treatment groups, except from the case where plasma glutamine was measured. This also suggests that the variability in the spaghetti plots for the placebo groups is comparably acceptable.

3.5 Summary of Findings and Discussion

In the present chapter post-absorptive patterns of five plasma amino acids after five challenges (citrulline, arginine, glutamine, placebo and 3-methylhistidine) were investigated. All results were presented in tables and figures. Results were satisfactory from robustness, despite an apparent variability. In other words, the
results are true and can be believed to then interpret them. Elements that provide robustness are:

1. Powerful enough design with no carryover effects for main outcomes (Tables 3.7 and 3.8).
2. Acceptable accurate standard curves when analysing data in mass spectrometer (Figures C.1–C.5).
3. Crossover ANOVA is reportedly robust (Driscoll, 1996).
4. Outlier analysis depicted markedly less influential points in the placebo treatment group in all plasma amino acids measured, except for plasma glutamine. In this case results should be treated with caution.

Returning to the original hypotheses formulated:

1. Citrulline can function successfully as an oral challenge test. This oral challenge test is time dependent with citrulline decreasing after the oral challenge is performed. Interestingly, citrulline was the most potent stimulator for all other amino acids, despite glutamine challenges being readily considered the most potent stimulators of citrulline production (Peters et al., 2007c; Peters et al., 2008b). It could be that this demonstrates more efficient amino acid absorption and that this could be an important step to countering disease-related catabolism or assist in protein anabolism. Hence, citrulline challenges could be useful in intestinal failure but also in liver failure where urea cycle pathways including glutamine, arginine and ornithine are implicated.

2. There were differences in the bioavailability of all five amino acids as shown with statistical testing (ANOVA $p < 0.05$ for citrulline, arginine and ornithine).

3. There was no period, carryover or sequence effect except for 3-methylhistidine which had a significant period effect (possibly due to small concentrations).

4. No treatment was equivalent to treatment with citrulline for producing equivalent post-absorptive concentrations ($p > 0.05$).

Citrulline challenges could be useful in intestinal failure but also in liver failure where urea cycle pathways including glutamine, arginine and ornithine are implicated. Limitations exist and include open-label design and short duration of follow-up. It was conducted in a small sample of healthy volunteers, who have different bioavailability than do patients when taken orally. Thus, the clinical significance of the findings – namely, challenges in patients for functional reason – must be clarified in additional studies involving patients with intestinal or liver
failure (Ouelaa et al., 2017; Sellmann et al., 2017). Furthermore, gastric emptying and intestinal transit factors were not measured in the present study and these could have been linked to citrulline challenges (Straathof et al., 2000; Bruins et al., 2004). Previous studies demonstrated that under stress during critical illness bowel motility was affected which was restored by administering L-arginine (Bruins et al., 2004), which however didn’t affect the motility of the lower oesophageal sphincter in healthy volunteers (Straathof et al., 2000).

The present study creates the potential for future studies in the field. These could involve:

1. A dynamic study with oral citrulline challenges in states of compromised intestine, such as extensive enteropathies (e.g. coeliac disease, mucositis, radiation, Crohn’s disease) or short bowel syndrome or intestinal failure.
2. Citrulline challenges in liver failure, since citrulline is part of the urea cycle.
3. Oral challenges in cases of compromised gut during critical illness. This is similar to extensive enteropathy but it is broader since multiple factors can contribute to critical illness enteropathy: endotoxemia, sepsis, catabolism, nitric oxide changes, arginine fluxes, presence of excessive lactate, etc. In Chapter 2 it was shown that citrulline was measured in critical illness but mainly as a static measure, with all the relevant shortcomings. A study in this setting would examine the potential of an oral challenge against single or serial measurements of citrulline.
3.6 **Tables**

**Table 3.1.** An example of the Williams design for a four-treatment, four-period cross-over trial.

<table>
<thead>
<tr>
<th>Subject no</th>
<th>Sequence no</th>
<th>Actual sequence</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>A-B-C-D</td>
<td>A B C D</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>B-D-A-C</td>
<td>B D A C</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>C-A-D-B</td>
<td>C A D B</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>D-C-B-A</td>
<td>D C B A</td>
</tr>
</tbody>
</table>

**Table 3.2.** Schedule of evaluations and procedures.

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Prior to screening</th>
<th>Screening</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>End of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit Number:</td>
<td>Study Day</td>
<td>SV V1 V2</td>
<td>V3 V4</td>
<td>V5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Informed consent</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eligibility criteria</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical History, Demographics</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical Examination(^b)</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concomitant medication(^c)</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine pregnancy test</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vital Signs</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inserting intravenous cannula</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood sample before 1st loading</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(considered as time 0)</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral loading</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood samples at 0.25, 0.5, 0.75, 1, 1.5, 2.3, 6h after the load</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine samples</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brief Medical History at end of visit and removal of cannula</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine samples of 16 and 24h post-administration</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brief Medical History via phone</td>
<td>X</td>
<td>X</td>
<td>X X X X</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after end of study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

\(^a\) Informed Consent must be signed before any screening visit assessments will be done.

\(^b\) Full physical examination (gastrointestinal, cardiovascular, respiratory, general appearance).

\(^c\) At screening, information on all medications taken in the previous 30 days will be collected.

\(^d\) This information will be identical with the screening visit, if the participant passes screening.
Table 3.3. A Williams Design for the Cross-over Trial. The number of treatments in this trial = 5.

<table>
<thead>
<tr>
<th>Sequence No</th>
<th>Period1</th>
<th>Period2</th>
<th>Period3</th>
<th>Period4</th>
<th>Period5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Placebo</td>
<td>3-MH</td>
<td>Gln</td>
<td>Arg</td>
<td>Cit</td>
</tr>
<tr>
<td>2</td>
<td>Gln</td>
<td>Placebo</td>
<td>Cit</td>
<td>3-MH</td>
<td>Arg</td>
</tr>
<tr>
<td>3</td>
<td>Cit</td>
<td>Gln</td>
<td>Arg</td>
<td>Placebo</td>
<td>3-MH</td>
</tr>
<tr>
<td>4</td>
<td>Arg</td>
<td>Cit</td>
<td>3-MH</td>
<td>Gln</td>
<td>Placebo</td>
</tr>
<tr>
<td>5</td>
<td>3-MH</td>
<td>Arg</td>
<td>Placebo</td>
<td>Cit</td>
<td>Gln</td>
</tr>
<tr>
<td>6</td>
<td>Cit</td>
<td>Arg</td>
<td>Gln</td>
<td>3-MH</td>
<td>Placebo</td>
</tr>
<tr>
<td>7</td>
<td>Arg</td>
<td>3-MH</td>
<td>Cit</td>
<td>Placebo</td>
<td>Gln</td>
</tr>
<tr>
<td>8</td>
<td>3-MH</td>
<td>Placebo</td>
<td>Arg</td>
<td>Gln</td>
<td>Cit</td>
</tr>
<tr>
<td>9</td>
<td>Placebo</td>
<td>Gln</td>
<td>3-MH</td>
<td>Cit</td>
<td>Arg</td>
</tr>
<tr>
<td>10</td>
<td>Gln</td>
<td>Cit</td>
<td>Placebo</td>
<td>Arg</td>
<td>3-MH</td>
</tr>
</tbody>
</table>

Table 3.4. The Randomisation schedule for the trial. The number of treatments in this trial = 5.

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>Arg--3-MH--Cit--Placebo--Gln</td>
</tr>
<tr>
<td>002</td>
<td>3-MH--Arg--Placebo--Cit--Gln</td>
</tr>
<tr>
<td>003</td>
<td>Cit--Gln--Arg--Placebo--3-MH</td>
</tr>
<tr>
<td>004</td>
<td>Cit--Arg--Gln--3-MH--Placebo</td>
</tr>
<tr>
<td>005</td>
<td>Placebo--3-MH--Gln--Arg--Cit</td>
</tr>
<tr>
<td>006</td>
<td>Gln--Placebo--Cit--3-MH--Arg</td>
</tr>
<tr>
<td>007</td>
<td>Arg--Cit--3-MH--Gln--Placebo</td>
</tr>
<tr>
<td>008</td>
<td>Gln--Cit--Placebo--Arg--3-MH</td>
</tr>
<tr>
<td>009</td>
<td>3-MH--Placebo--Arg--Gln--Cit</td>
</tr>
<tr>
<td>010</td>
<td>Placebo--Gln--3-MH--Cit--Arg</td>
</tr>
<tr>
<td>011</td>
<td>3-MH--Placebo--Arg--Gln--Cit</td>
</tr>
<tr>
<td>012</td>
<td>Arg--3-MH--Cit--Placebo--Gln</td>
</tr>
<tr>
<td>013</td>
<td>Gln--Cit--Placebo--Arg--3-MH</td>
</tr>
<tr>
<td>014</td>
<td>3-MH--Arg--Placebo--Cit--Gln</td>
</tr>
<tr>
<td>015</td>
<td>Cit--Gln--Arg--Placebo--3-MH</td>
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<tr>
<td>016</td>
<td>Cit--Arg--Gln--3-MH--Placebo</td>
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<tr>
<td>018</td>
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<tr>
<td>019</td>
<td>Placebo--3-MH--Gln--Arg--Cit</td>
</tr>
<tr>
<td>020</td>
<td>Placebo--Gln--3-MH--Cit--Arg</td>
</tr>
</tbody>
</table>
Table 3.5. General descriptive statistics regarding arginine, citrulline, glutamine, ornithine, and 3-methylhistidine concentrations (μmol/L).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Variance</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine Concentration</td>
<td>900</td>
<td>153.8219</td>
<td>4.02715</td>
<td>120.8146</td>
<td>706.94</td>
<td>724.2</td>
<td>14596.16</td>
<td>1.986</td>
<td>0.082</td>
</tr>
<tr>
<td>Citrulline Concentration</td>
<td>900</td>
<td>115.49</td>
<td>5.15092</td>
<td>154.5275</td>
<td>835.63</td>
<td>840.3</td>
<td>23878.74</td>
<td>1.818</td>
<td>0.082</td>
</tr>
<tr>
<td>Glutamine Concentration</td>
<td>900</td>
<td>534.1462</td>
<td>8.06871</td>
<td>242.0614</td>
<td>1023.72</td>
<td>1066.87</td>
<td>58593.7</td>
<td>0.126</td>
<td>0.082</td>
</tr>
<tr>
<td>Ornithine Concentration</td>
<td>900</td>
<td>119.6382</td>
<td>3.32826</td>
<td>99.8479</td>
<td>431.58</td>
<td>435.26</td>
<td>9969.603</td>
<td>1.738</td>
<td>0.082</td>
</tr>
<tr>
<td>3-MH Concentration</td>
<td>900</td>
<td>4.4797</td>
<td>0.04579</td>
<td>1.37376</td>
<td>6.48</td>
<td>6.67</td>
<td>1.887</td>
<td>-0.756</td>
<td>0.082</td>
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</table>
### Table 3.6. Means of AUC, \(C_{\text{max}}\) and TOMC per treatment for plasma amino acids arginine, citrulline, glutamine, ornithine, and 3-methylhistidine.

<table>
<thead>
<tr>
<th>Plasma Amino Acid measured</th>
<th>Citrulline</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Placebo</th>
<th>3-Methyl-Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Arginine</td>
<td>20</td>
<td>1675.51</td>
<td>865.71</td>
<td>13023</td>
<td>10563</td>
</tr>
<tr>
<td>Citrulline</td>
<td>20</td>
<td>2453.44</td>
<td>2105.15</td>
<td>2866.79</td>
<td>2587.27</td>
</tr>
<tr>
<td>Glutamine</td>
<td>20</td>
<td>2819.73</td>
<td>1331.37</td>
<td>4685.81</td>
<td>3795.98</td>
</tr>
<tr>
<td>Ornithine</td>
<td>20</td>
<td>2411.77</td>
<td>922.94</td>
<td>4033.12</td>
<td>1058.55</td>
</tr>
<tr>
<td>3-Methyl-Histidine</td>
<td>20</td>
<td>2769.77</td>
<td>836.80</td>
<td>3082</td>
<td>2621</td>
</tr>
<tr>
<td><strong>(C_{\text{max}})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>20</td>
<td>3425.7</td>
<td>1800.15</td>
<td>680.48</td>
<td>2662.15</td>
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<tr>
<td>Citrulline</td>
<td>20</td>
<td>4550.6</td>
<td>4404.57</td>
<td>840.3</td>
<td>809.61</td>
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<td>Glutamine</td>
<td>20</td>
<td>706.3</td>
<td>3103.12</td>
<td>1366.7</td>
<td>681.62</td>
</tr>
<tr>
<td>Ornithine</td>
<td>20</td>
<td>4477.2</td>
<td>1066.24</td>
<td>4353.26</td>
<td>2356</td>
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<tr>
<td>3-Methyl-Histidine</td>
<td>20</td>
<td>491.17</td>
<td>2.65</td>
<td>484.24</td>
<td>1172</td>
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<tr>
<td><strong>TOMC</strong></td>
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<td></td>
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<td>Arginine</td>
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<td>239.08</td>
<td>0.05</td>
<td>0.01</td>
<td>0.10</td>
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<td>Citrulline</td>
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<td>1.39</td>
<td>0.25</td>
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<td>Glutamine</td>
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<td>344.21</td>
<td>0.6</td>
<td>2.96</td>
<td>2.46</td>
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<td>Ornithine</td>
<td>20</td>
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<td>0.5</td>
<td>6.18</td>
<td>1.52</td>
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<td>174.14</td>
<td>0.25</td>
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</table>
Table 3.7. Crossover ANOVA for AUC.

<table>
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<tr>
<th>Source of Variation</th>
<th>CITRULLINE</th>
<th>ARGinine</th>
<th>GLUTAMINE</th>
<th>ORNITHINE</th>
<th>3-METHYL-HISTIDINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partial SS</td>
<td>df</td>
<td>MS</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Intersubjects</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequence effect</td>
<td>72446.37</td>
<td>9</td>
<td>8049.6</td>
<td>0.62</td>
<td>0.76</td>
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<tr>
<td>Residuals</td>
<td>130497.52</td>
<td>10</td>
<td>13049.75</td>
<td>0.75</td>
<td>0.6783</td>
</tr>
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<td>Intrasubjects</td>
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<td></td>
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</tr>
<tr>
<td>Treatment effect</td>
<td>7.13E+07</td>
<td>4</td>
<td>1.78E+07</td>
<td>1020.43</td>
<td>0.62</td>
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<tr>
<td>Carryover effect</td>
<td>42336.77</td>
<td>4</td>
<td>10584.19</td>
<td>0.61</td>
<td>0.6599</td>
</tr>
<tr>
<td>Period effect</td>
<td>64058.35</td>
<td>4</td>
<td>16014.59</td>
<td>0.92</td>
<td>0.4596</td>
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<td>17476.63</td>
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<tr>
<td>Total</td>
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<td>Intersubjects</td>
<td></td>
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</tr>
<tr>
<td>Sequence effect</td>
<td>1.07E+07</td>
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<td>1.18E+06</td>
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<td>0.8827</td>
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<tr>
<td>Residuals</td>
<td>2.68E+07</td>
<td>10</td>
<td>2.68E+06</td>
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<tr>
<td>Treatment effect</td>
<td>5.42E+06</td>
<td>4</td>
<td>1.36E+06</td>
<td>1</td>
<td>0.4118</td>
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<tr>
<td>Carryover effect</td>
<td>6.94E+06</td>
<td>4</td>
<td>1.74E+06</td>
<td>1.29</td>
<td>0.2843</td>
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<td>1.97E+06</td>
<td>1.46</td>
<td>0.2243</td>
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<td>Residuals</td>
<td>9.18E+06</td>
<td>68</td>
<td>1.35E+06</td>
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<tr>
<td>Total</td>
<td>1.54E+08</td>
<td>99</td>
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</tbody>
</table>

Note for all models: Omnibus measure of separability of treatment and carryover = 77.6393%
### Table 3.8. Crossover ANOVA for C\textsubscript{max}.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>CITRULLINE</th>
<th>ARGinine</th>
<th>GLUTAMINE</th>
<th>ORNITHINE</th>
<th>3-METHYL-HISTIDINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partial SS</td>
<td>df</td>
<td>MS</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td><strong>Intersubjects</strong></td>
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<td></td>
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</tr>
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<td>0.9564</td>
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<tr>
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<td>1958.46</td>
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<td></td>
</tr>
<tr>
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<td>734320.3</td>
<td>299.35</td>
<td>&lt;0.001</td>
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<td>0.2618</td>
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<td>1667.01</td>
<td>0.68</td>
<td>0.6085</td>
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<tr>
<td>Residuals</td>
<td>19584.58</td>
<td>10</td>
<td>1958.46</td>
<td>0.8</td>
<td>0.6305</td>
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<tr>
<td><strong>Total</strong></td>
<td>3.30E+06</td>
<td>99</td>
<td>2.59E+06</td>
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</tbody>
</table>

Note for all models: Omnibus measure of separability of treatment and carryover = 77.6393%
Table 3.9. Multiple comparisons between mean AUC (Tukey). Differences express (I-J). * The mean difference is significant at the 0.05 level.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>(J) Treatment</th>
<th>(I) Treatment</th>
<th>Citrulline</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-6} for Arginine</td>
<td>Arginine</td>
<td>268.3</td>
<td>268.3</td>
<td>268.3</td>
<td>268.3</td>
<td>268.3</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>912.6*</td>
<td>644.4*</td>
<td>644.4*</td>
<td>644.4*</td>
<td>644.4*</td>
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<tr>
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<td>Placebo</td>
<td>1103.8*</td>
<td>835.6*</td>
<td>835.6*</td>
<td>835.6*</td>
<td>835.6*</td>
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<tr>
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<td>3-Methylhistidine</td>
<td>1053.0*</td>
<td>784.7*</td>
<td>784.7*</td>
<td>784.7*</td>
<td>784.7*</td>
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<tr>
<td>AUC_{0-6} for Citrulline</td>
<td>Arginine</td>
<td>2195.1*</td>
<td>2195.1*</td>
<td>2195.1*</td>
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<td>3-Methylhistidine</td>
<td>2206.4*</td>
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<td>11.2</td>
<td>11.2</td>
<td>11.2</td>
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<tr>
<td>AUC_{0-6} for Glutamine</td>
<td>Arginine</td>
<td>-560.3</td>
<td>-560.3</td>
<td>-560.3</td>
<td>-560.3</td>
<td>-560.3</td>
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<tr>
<td></td>
<td>Glutamine</td>
<td>-663.1</td>
<td>-102.9</td>
<td>-102.9</td>
<td>-102.9</td>
<td>-102.9</td>
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<tr>
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<td>569.9</td>
<td>569.9</td>
<td>569.9</td>
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<tr>
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<td>3-Methylhistidine</td>
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<td>Arginine</td>
<td>395.9*</td>
<td>395.9*</td>
<td>395.9*</td>
<td>395.9*</td>
<td>395.9*</td>
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<td></td>
<td>Glutamine</td>
<td>997.4*</td>
<td>601.4*</td>
<td>601.4*</td>
<td>601.4*</td>
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<td>1088.5*</td>
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<td>692.6*</td>
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<td>1039.1*</td>
<td>643.2*</td>
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<td>1.5</td>
<td>1.5</td>
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Table 3.10. Test: reference values for AUC. Anderson Hauck test $p$-value < 0.05 indicates equivalence.

<table>
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<tr>
<th>Treatment</th>
<th>Citrulline</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
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<td><strong>Citrulline concentration</strong></td>
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</tr>
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<td>Arginine</td>
<td>70.31% to 71.19% ($p &lt; 0.05$)</td>
<td>58.47% to 58.83% ($p &lt; 0.05$)</td>
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<tr>
<td>Glutamine</td>
<td>70.43% to 71.06% ($p &lt; 0.05$)</td>
<td>57.95% to 59.36% ($p &lt; 0.05$)</td>
<td>60.83% to 61.83% ($p &lt; 0.05$)</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>70.51% to 70.98% ($p &lt; 0.05$)</td>
<td>58.18% to 59.12% ($p &lt; 0.05$)</td>
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</tr>
<tr>
<td>3-Methylhistidine</td>
<td>70.54% to 70.96% ($p &lt; 0.05$)</td>
<td>58.18% to 59.12% ($p &lt; 0.05$)</td>
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<tr>
<td><strong>Arginine concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>96.61% to 99.11% ($p = 0.752$)</td>
<td>97.23% to 98.40% ($p = 0.799$)</td>
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<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>96.73% to 98.99% ($p = 0.759$)</td>
<td>97.38% to 98.25% ($p = 0.759$)</td>
<td>97.38% to 98.72% ($p = 0.759$)</td>
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<tr>
<td>Placebo</td>
<td>96.83% to 98.88% ($p = 0.763$)</td>
<td>97.56% to 98.07% ($p = 0.794$)</td>
<td>96.69% to 98.51% ($p = 0.777$)</td>
<td>96.65% to 98.26% ($p = 0.760$)</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>96.42% to 99.30%</td>
<td>97.56% to 98.07% ($p = 0.794$)</td>
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<td></td>
</tr>
<tr>
<td><strong>Glutamine concentration</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Arginine</td>
<td>101.09% to 102.45%</td>
<td>101.30% to 102.18% ($p = 0.775$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>100.70% to 102.85% ($p = 0.763$)</td>
<td>101.06% to 102.43%</td>
<td>100.62% to 102.85% ($p = 0.763$)</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>101.50% to 102.05% ($p = 0.789$)</td>
<td>101.03% to 102.45% ($p = 0.752$)</td>
<td>100.69% to 102.78% ($p = 0.762$)</td>
<td>101.05% to 102.55% ($p = 0.786$)</td>
</tr>
<tr>
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<td>100.82% to 102.73% ($p = 0.747$)</td>
<td>101.03% to 102.45% ($p = 0.752$)</td>
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<td></td>
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<tr>
<td><strong>Ornithine concentration</strong></td>
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</tbody>
</table>
### Chapter 3: Pharmacodynamic Study

#### Treatment

<table>
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<tr>
<th>Treatment</th>
<th>Citrulline</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>94.16% to 96.03% ((p = 0.764))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>93.74% to 95.94% ((p = 0.795))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
</tr>
<tr>
<td>Glutamine</td>
<td>94.2% to 95.99% ((p = 0.737))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>93.70% to 95.98% ((p = 0.771))</td>
<td>92.86% to 95.35%</td>
</tr>
<tr>
<td>Placebo</td>
<td>94.92% to 95.27% ((p = 0.767))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>93.70% to 95.98% ((p = 0.771))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
</tr>
</tbody>
</table>

#### 3-Methylhistidine concentration

<table>
<thead>
<tr>
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<th>Citrulline</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>96.68% to 100.36%</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>96.20% to 100.80%</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
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<tr>
<td>Glutamine</td>
<td>96.75% to 100.29% ((p = 0.752))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>93.49% to 94.71% ((p = 0.765))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
</tr>
<tr>
<td>Placebo</td>
<td>97.21% to 99.84% ((p = 0.740))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>96.37% to 100.68% ((p = 0.771))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>95.38% to 101.67% ((p = 0.772))</td>
<td>Anderson Hauck test (p)-value: &lt; 0.05</td>
<td>97.17% to 99.83% ((p = 0.804))</td>
<td>95.30 to 101.70% ((p = 0.780))</td>
</tr>
</tbody>
</table>

Anderson Hauck test \(p\)-value: < 0.05
Table 3.11. Heteroscedasticity tests and influential observations according to Cook’s distance.

<table>
<thead>
<tr>
<th>Plasma Citrulline AUC</th>
<th>Plasma Arginine AUC</th>
<th>Plasma Glutamine AUC</th>
<th>Plasma Ornithine AUC</th>
<th>Plasma 3-Methylhistidine AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breusch-Pagan / Cook-Weisberg test for Heteroscedasticity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>44.78</td>
<td>44.63</td>
<td>0.15</td>
<td>42.78</td>
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<tr>
<td>$p$</td>
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<td>&lt; 0.001</td>
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Cook’s Influential Observations

<table>
<thead>
<tr>
<th>ID</th>
<th>Treatment</th>
<th>Cook’s D</th>
<th>ID</th>
<th>Treatment</th>
<th>Cook’s D</th>
<th>ID</th>
<th>Treatment</th>
<th>Cook’s D</th>
<th>ID</th>
<th>Treatment</th>
<th>Cook’s D</th>
</tr>
</thead>
<tbody>
<tr>
<td>S09</td>
<td>Citrulline</td>
<td>0.044265</td>
<td>S15</td>
<td>Citrulline</td>
<td>0.16398</td>
<td>S21</td>
<td>Citrulline</td>
<td>0.044138</td>
<td>S02</td>
<td>Citrulline</td>
<td>0.061632</td>
</tr>
<tr>
<td>S10</td>
<td>Citrulline</td>
<td>0.087887</td>
<td>S16</td>
<td>Citrulline</td>
<td>0.11762</td>
<td>S10</td>
<td>Arginine</td>
<td>0.072873</td>
<td>S09</td>
<td>Glutamine</td>
<td>0.073003</td>
</tr>
<tr>
<td>S12</td>
<td>Citrulline</td>
<td>0.06152</td>
<td>S19</td>
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<td>0.047331</td>
<td>S23</td>
<td>Arginine</td>
<td>0.040662</td>
<td>S12</td>
<td>Citrulline</td>
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<tr>
<td>S18</td>
<td>Citrulline</td>
<td>0.150955</td>
<td>S23</td>
<td>Citrulline</td>
<td>0.084224</td>
<td>S13</td>
<td>Glutamine</td>
<td>0.069266</td>
<td>S13</td>
<td>Citrulline</td>
<td>0.058716</td>
</tr>
<tr>
<td>S20</td>
<td>Citrulline</td>
<td>0.041899</td>
<td>S15</td>
<td>Arginine</td>
<td>0.056279</td>
<td>S20</td>
<td>Glutamine</td>
<td>0.070475</td>
<td>S14</td>
<td>Citrulline</td>
<td>0.042913</td>
</tr>
<tr>
<td>S21</td>
<td>Citrulline</td>
<td>0.070912</td>
<td>S23</td>
<td>Arginine</td>
<td>0.095587</td>
<td>S09</td>
<td>Placebo</td>
<td>0.058897</td>
<td>S17</td>
<td>Citrulline</td>
<td>0.0773</td>
</tr>
<tr>
<td>S22</td>
<td>Citrulline</td>
<td>0.045516</td>
<td>S21</td>
<td>Glutamine</td>
<td>0.049899</td>
<td>S12</td>
<td>Placebo</td>
<td>0.072856</td>
<td>S18</td>
<td>Citrulline</td>
<td>0.051824</td>
</tr>
<tr>
<td>S23</td>
<td>Citrulline</td>
<td>0.046197</td>
<td>S16</td>
<td>3-Methyl-H</td>
<td>0.04345</td>
<td>S20</td>
<td>Placebo</td>
<td>0.052024</td>
<td>S23</td>
<td>Citrulline</td>
<td>0.072304</td>
</tr>
<tr>
<td>S10</td>
<td>Arginine</td>
<td>0.054543</td>
<td>S23</td>
<td>3-Methyl-H</td>
<td>0.078569</td>
<td>S02</td>
<td>Arginine</td>
<td>0.119699</td>
<td>S15</td>
<td>Arginine</td>
<td>0.04179</td>
</tr>
<tr>
<td>S12</td>
<td>Glutamine</td>
<td>0.074601</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>S18</td>
<td>Arginine</td>
<td>0.040249</td>
</tr>
<tr>
<td>S14</td>
<td>Glutamine</td>
<td>0.044515</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S22</td>
<td>Arginine</td>
<td>0.135838</td>
</tr>
</tbody>
</table>

ID: Subject ID; Treatment: Amino Acid ingested; Cook’s D: Cook’s Distance; 3-Methyl-H: 3-methylhistidine.
3.7 Figures

Figure 3.1. Study diagram. Example of the ten sequences of amino acids the subjects received.
Figure 3.2. Citrulline concentrations post ingestion of citrulline.
Figure 3.3. Arginine concentrations post ingestion of citrulline.
Figure 3.4. Glutamine concentrations post ingestion of citrulline.
Figure 3.5. Ornithine concentrations post ingestion of citrulline.
Figure 3.6. 3-Methylhistidine concentrations post ingestion of citrulline.
Figure 3.7. Citrulline concentrations post ingestion of arginine.
Figure 3.8. Arginine concentrations post ingestion of arginine.
Figure 3.9. Glutamine concentrations post ingestion of arginine.
Figure 3.10. Ornithine concentrations post ingestion of arginine.
Figure 3.11. 3-Methylhistidine concentrations post ingestion of arginine.
Figure 3.12. Citrulline concentrations post ingestion of glutamine.
Figure 3.13. Arginine concentrations post ingestion of glutamine.
Figure 3.14. Glutamine concentrations post ingestion of glutamine.
Figure 3.15. Ornithine concentrations post ingestion of glutamine.
Figure 3.16. 3-Methylhistidine concentrations post ingestion of glutamine.
Figure 3.17. Citrulline concentrations post ingestion of placebo.
Figure 3.18. Arginine concentrations post ingestion of placebo.
Figure 3.19. Glutamine concentrations post ingestion of placebo.
Figure 3.20. Ornithine concentrations post ingestion of placebo.
Figure 3.21. 3-Methylhistidine concentrations post ingestion of placebo.
Figure 3.22. Citrulline concentrations post ingestion of 3-methylhistidine.
Figure 3.23. Arginine concentrations post ingestion of 3-methylhistidine.
Figure 3.24. Glutamine concentrations post ingestion of 3-methylhistidine.
Figure 3.25. Ornithine concentrations post ingestion of 3-methylhistidin.
Figure 3.26. 3-Methylhistidine concentrations post ingestion of 3-methylhistidine.
Figure 3.27. Boxplots for AUC, $C_{\text{max}}$ and TOMC.
Figure 3.28. DFBETAs for the AUC of each plasma amino acid measured and per treatment.
Chapter 4
Quality of Life in Short Bowel Syndrome Patients

4.1 Introduction

As defined in the previous chapter, intestinal failure occurs when there is reduced intestinal absorption so that macronutrient and/or water and electrolyte supplements are needed to maintain health and/or growth. Short bowel syndrome is a global malabsorption syndrome due to insufficient absorptive capacity and/or disturbed gastrointestinal regulation resulting from extensive small bowel resections. Short bowel syndrome may occur after resection of more than 50% and is obligatory after resection of more than 70% of the small intestine or if less than 100 cm of small bowel are left. It is particularly severe after resection of the ileocecal region or if the colon has been removed additionally (Pironi et al., 2015; Pironi et al., 2016). Apart from global malnutrition, clinical symptoms may be caused by specific deficiencies depending on the site of intestinal loss (Keller et al., 2004; Allan and Lal, 2018).

There are three main types of patient with a short bowel: those who have had a jejunooileal resection and a jejunocolic anastomosis (jejunum-colon); those who have had a predominantly jejunal resection, and have more than 10 cm of terminal ileum and the colon remaining (jejunum-ileum); and those who have had a jejunooileal resection, colectomy, and formation of a stoma (jejunostomy). The most common reasons for a short bowel in adults are Crohn’s disease, superior mesenteric artery thrombosis, and irradiation damage (Keller et al., 2004; Nightingale and Woodward, 2006; Pironi et al., 2015; Pironi et al., 2016).

The previous chapters discussed citrulline as a diagnostic marker in intestinal diseases and oral challenges of citrulline and other amino acids in healthy volunteers as a proxy of intestinal function. The present chapter will attempt to infer whether citrulline can be used as a proxy of quality of life in patients with short bowel syndrome. This is an interesting question that requires some thought. Citrulline essentially reflects enterocyte mass, disease severity and absorption (see Chapter 2). It is reasonable to think that when citrulline is abnormal, the intestine is not fully functioning and hence this diseased state may possibly lead to impaired
quality of life. Hence, from a clinical point of view, measuring citrulline in daily practice, might reflect the associated level of quality life in short bowel syndrome patients, functioning as a surrogate marker for quality of life. Nevertheless the literature of quality of life in short bowel syndrome is quite variable. Below I review the literature in this field and describe results of a quality of life study performed at University College London Hospitals. In the discussion, I will come back to the question inferred above regarding citrulline as a surrogate marker of quality of life.

### 4.2 Quality of Life in Short Bowel Syndrome Patients

An important aspect of quality of life is nutrition. Ljungqvist and Pichard (2008) comment on this and say:

> Just because a person becomes a patient should not result in him or her losing the right to enjoy meals as we otherwise would in everyday life. It is, however, striking how often this fundamental and basic right is lost and not fulfilled in the midst of plentiful medical prescriptions and treatments. Despite the obvious insight of the fundamentals of food, the medical profession and caregivers in other situations fail to come to the insight that nutrition is one of the keys to successful outcomes and a better quality of life (p. 320).

Nevertheless, studies on quality of life in patients with short bowel syndrome could be more (Jeppesen et al., 1999; Carlsson et al., 2003b, a; Huisman-de Waal et al., 2007; Kalaitzakis et al., 2008). Short bowel syndrome is a disease that burdens patients’ daily lives with intense therapies, complications needing frequent hospital admissions, stressful events, professional challenges, personal challenges and concomitant psychological repercussions. In addition to this, patients have particular aspects to their quality specific for their disease. For example, cancer operations that lead to short bowel syndrome burden patients with additional issues of quality of life related to their cancer (e.g. prognosis and morbidity) (Naghibi et al., 2015; Cotogni et al., 2017).

Overall, patients on with short bowel syndrome seem to report lower quality of life compared with the healthy population and quality of life seems to decline with longer parenteral nutrition-dependency (Avitzur and Miserachs, 2018). Various studies have identified over time a group of main issues that concern patients with short bowel syndrome (Baxter et al., 2010; Winkler, 2010; Wilburn et al., 2017;
Avitzur and Miserachs, 2018). These are shown in Table 4.1. A discussion on certain main studies in the field is given below.

Kalaitzakis et al. (2008) used four validated questionnaires to measure aspects of quality of life [Short Form 36 (SF-36)], psychological distress (hospital anxiety and depression scale), fatigue (fatigue impact scale), and gastrointestinal symptoms (gastrointestinal symptom rating scale) in 26 out of 28 patients (93%) attending a short bowel syndrome clinic (median age 62 years, 15 females) at a tertiary referral centre. The control group were people from the general population (n = 286) as well as patients with inflammatory bowel disease (n = 41). Their results showed that short bowel syndrome patients had significantly lower SF-36 physical and mental component summaries than those in the general population as well as significantly lower SF-36 physical but not mental component summaries compared with those of inflammatory bowel disease patients. Fatigue and gastrointestinal symptoms were more severe in short bowel syndrome patients than in inflammatory bowel disease patients (p < 0.05). Their conclusion was that patients with short bowel syndrome show poor quality of life compared with that in the general population with fatigue and gastrointestinal symptoms being more severe in patients with short bowel syndrome.

Carlsson et al. (2003b) studied 28 short bowel syndrome patients (19 females), mean age 54 years, with eight of them being on home parenteral nutrition. Quality of life was recorded using a visual analogue scale and health-related quality of life was assessed using SF-36 and compared with matched controls. Concerns were assessed using the Rating Form of Inflammatory bowel disease patient concerns. Coping strategies were investigated using the Jalowiec coping scale (Jalowiec et al., 1984) Their results showed that patients’ greatest concerns were fear of being a burden (median = 81), having surgery (median = 60) and loss of energy (median = 49). Health-related quality of life was significantly reduced compared to controls (p < 0.05), while patients’ receiving home parenteral nutrition rated quality of life lower than those without home parenteral nutrition. The presence of a stoma appeared not to influence quality of life negatively but stoma patients expressed more concern. Confrontational coping style was most frequently used.

An important study in the field of short bowel syndrome looked at how the presence of home parenteral nutrition affected patient’s daily lives (Baxter et al., 2010). Their
inclusion criterion were patients on home parenteral nutrition and included other causes of intestinal failure in addition to short bowel syndrome. Their sample included 49 patients with short bowel syndrome (out of 100 in total). The authors developed a questionnaire that comprised of 48 items examining body image, coping, ability to eat or drink, employment, emotional problems, fatigue, general health, gastrointestinal symptoms, ability to go on holiday or travel, immobility, pain, physical role, social function, sleep pattern, sexual function, vitality, worry, and weight. These concepts were generally reduced in this patient group.

Olieman et al. (2012) examined health related quality of life in children with a history of infantile short bowel syndrome treated in their first year of life. Thirty-one children were included (19 girls, mean age 12 yeas). Their average health related quality of life was significantly lower than that of a control sample of 275 healthy age-matched children and their parents ($p < 0.05$). Physical and school functioning were significantly lower in the children with short bowel syndrome compared to controls and there was also emotional impact on the parents of these children compared to their respective parent controls.

Hence, quality of life is severely affected in patients with short bowel syndrome. Although the there is a literature examining these aspects, multiple aspects are currently unexplored. Namely, there isn’t an established quantitative tool that allows to measure the impact of the presence of short bowel syndrome on the quality of life in this group of patients. In pursue of this aim, a questionnaire was developed by a group in Germany and had not been fully validated at the time of the present study (2011-2012). The validation was later published inclusive of our results (Berghöfer et al., 2013). Hence, the aims of the present study were:

1. Investigate the psychometric properties of a newly developed quality of life measure, specific for short bowel syndrome patients, with patients from University College London Hospital.

2. Investigate the main aspects that affect the quality of life in patients with short bowel syndrome.
4.3 University College London Hospital Study

4.3.1 Methods

A short bowel syndrome quality of life scale (SBSQoL™) containing 17 questions with answers on a visual analogue scale (SBSQoL1-17) was used (Table 4.2). This questionnaire has been developed by a group in Germany and wasn’t fully validated at the time of the present study (2011-2012). The validation was later published inclusive of our results (Berghöfer et al., 2013).

The answers of this scale were transformed into a 10-point Likert type, where 1 and 10 means ‘not at all’ and ‘very much’ respectively. Only SBSQoL1 went from ‘excellent’ to ‘unbearably bad’. The psychometric properties of the questionnaire (reliability and internal consistency) were examined and exploratory factor analysis was performed in order to examine if the questions could be reduced to a small number of homogeneous factors. Reliability was assessed with Cronbach’s alpha (Tsagris et al., 2013). The differences between patients belonging to the upper and lower 25% percentile group of SBSQoL1, which was taken as a general proxy of quality of life, was also examined. All statistical computations were done with SPSS 22.0.

4.3.2 Results

The sample included 15 short bowel syndrome subjects (12 females, 3 males), mean age: 50.7 ± 19.8 years (range 18-88). For SBSQoL1 the mean (± SD) was 4.8 ± 2.7. For all other questions, the mean ranged from 6.1-7.7. Results are shown in Figure 4.1.

Reliability and internal consistency measures of the overall scale were excellent (Cronbach’s alpha: 0.907, Standardised Cronbach’s alpha: 0.910, Spearman-Brown coefficient: 0.923, Guttman split-half coefficient: 0.920). Exploratory factor analysis was performed with principal component analysis, which yielded four factors, explaining 80% of variance; the scree plot image confirmed this (Figure 4.2). Factor loadings are shown in Table 4.3. The four factors were labelled as:

Factor 1 ‘General Physical Symptoms and Activities’ (11 questions)

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1 Factor analysis usually requires a sample over 50. One recent study though (de Winter et al., 2009) allow for a sample size under 50 for high communalities, which is demonstrated in our sample (Table 4.2).
Factor 2 ‘Daily activities’ (3 questions)

Factor 3 ‘Digestive System Symptoms’ (2 questions)

Factor 4 ‘Stoma Related Symptoms’ (1 question)

Mann-Whitney U test was employed to examine differences in questions SBSQoL2-17 between the two percentiles mentioned above; the sample was small and SBSQoL2-17 were not normally distributed (Kolmogorov-Smirnov test was significant for all) (Table 4.4).

A significant difference was detected only in questions 14 (fatigue) and 15 (diarrhoea/increased stomal output). The upper 25% percentile group had higher medians in their answers, while there were borderline differences in questions 12 (sleep), 13 (gastroenterological symptoms), and 16 (musculoskeletal symptoms).

Results are shown in Figure 4.3 and Table 4.5.

4.4 Summary and Conclusion

The present study investigated quality of life in patients with short bowel syndrome. The SBSQoL™ scale is highly reliable in measuring quality of life in short bowel syndrome patients with a high Cronbach’s alpha. This was also demonstrated in the larger sample, of which the present study was part of (Berghöfer et al., 2013). The differences with the results from the larger study were the number of factors extracted from exploratory factor analysis. Two factors were extracted from the larger study, factor 1 comprised of 11 items and factor of 6 items. The study demonstrated that SBSQoL™ had satisfactory test re-test reliability, responsiveness and construct validity (Berghöfer et al., 2013). The same group proceeded to test whether the growth factor teduglutide would improve quality of life in patients with short bowel syndrome since it decreased the need for parenteral nutrition to a certain degree (Jeppesen et al., 2013). The authors demonstrated significant decrease in the total scale score, factor 1 score and factor 2 score in patients who had received teduglutide. However, when this decrease was compared to placebo, the difference was non-significant. This was attributed to a short observation period, imbalances in oral fluid intake in relation to parenteral solution reductions, large patient and effect heterogeneity and occurrence of gastrointestinal adverse events in a subgroup of teduglutide-treated patients may account (Jeppesen et al., 2013).
The next important finding of the present study was that the main causes of low quality of life were fatigue, diarrhoea/increased stomal output, lack of sleep, gastrointestinal symptoms, and muscle pains. Lack of sleep has been frequently associated with short bowel syndrome predominantly due to long parenteral fluid infusion times and/or a daily infusion schedule because of large-volume fluid requirements, due to pumps and equipment alarms, fear of catheter dislodgement, nocturia, polyuria, need for extra storage space, and complaints of carrying heavy backpacks (Winkler and Smith, 2014). Lack of sleep with associated macronutrient imbalances are frequently associated with fatigue, muscle pains and widespread pain syndromes. These in turn can also affect sleep negatively causing eventually functional impairment and even depression (Winkler, 2005, 2010; Winkler et al., 2010).

Physicians should treat these symptoms as much as possible to ease the burden of patients’ disease (e.g. loperamide for diarrhoea, paracetamol or stronger pain killers, zopiclone for sleeping). The importance of addressing these issues is related with better outcomes in patients with short bowel syndrome. Physical symptoms of fatigue, muscle pains, increase stoma output, metabolic bone disease, electrolyte, macronutrient and micronutrient imbalances can all lead to mental health disease, social and financial consequences which are known to decrease life expectancy in general (Marmot et al., 2012).

Next, I would like to address the initial hypothesis that citrulline could function as a surrogate marker of quality of life in patients with short bowel syndrome and by extension in other forms of intestinal failure. The present study demonstrated that quality of life is decreased in patients with short bowel syndrome. The larger study demonstrated that quality of life improved with teduglutide and reductions in parenteral solutions’ needs. In Chapter 2, it was shown that lower citrulline levels were a diagnostic marker for parenteral nutrition dependence in patients with short bowel syndrome and extensive mucosal disease. However, in the studies by Berghöfer et al. (2013) and Jeppesen et al. (2013), citrulline was not measured and correlated with quality of life. Nevertheless, due to the simultaneous association of quality of life and changes in citrulline levels with short bowel syndrome, it is reasonable to advocate based on the transitive property of associations that changes
of citrulline are hence associated with changes with quality of life in patients with short bowel syndrome.

The limitations of the present study include as small sample size, lack of repeated measures over time to assess for re-test reliability and lack of measuring other constructs to assess for construct validity. Another limitation is the inability to discriminate to the effect on quality of life by home parenteral nutrition (which most patients on short bowel are treated with) from reduction of quality of life by short bowel syndrome. There is an obvious overlap of effects of both aspects on quality of life, but ideally a questionnaire should be specific with regards to the patient group and characteristics. Despite these limitations, the similar results found in the present study with the larger study by Berghöfer et al. (2013) lends to support to the validity of the current findings.

Finally, the present study provides grounds for certain future studies that should take place. The present study intends to form the basis for investigation of quality of life in a larger sample. This larger study ideally should have repeated measures and associated citrulline measurements as well and be able to differentiate the consequences on quality of life from home parenteral nutrition and short bowel syndrome. Also, future intentions include examining whether the results of exploratory factor analysis can be used to transform the 17-item questionnaire into a 4-item short bowel syndrome quality of life scale (since four factors were identified in the factor analysis) which can be used very easily in clinical settings. A similar study took place recently, by which a patient preference-weighted scoring algorithm for the SBS-QoL™ was developed and was able to estimate a wide range of utility values from patient-level SBS-QoL™ data (Lloyd et al., 2014). This study essentially narrowed down the seventeen items of the initial scale to eight items relates to six health states. The authors advocated using the original scale for more accurate assessment of quality of life, but in the field of questionnaires and psychometric research, short forms of original larger scales is useful for patient reasons initially, who might not be willing to answer lengthy questionnaires, as well as practical reasons of design, sample size and analysis, ensuring powerful enough samples with less items if possible.
### 4.5 Tables

**Table 4.1.** Disease-related concerns for patients with short bowel syndrome.

<table>
<thead>
<tr>
<th>Concern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Being a burden on others</td>
</tr>
<tr>
<td>Having surgery</td>
</tr>
<tr>
<td>Energy level</td>
</tr>
<tr>
<td>Loss of sexual drive</td>
</tr>
<tr>
<td>Access to quality medical care</td>
</tr>
<tr>
<td>Pain or suffering</td>
</tr>
<tr>
<td>Dying early</td>
</tr>
<tr>
<td>Loss of bowel control</td>
</tr>
<tr>
<td>Feeling out of control</td>
</tr>
<tr>
<td>Uncertain nature of disease</td>
</tr>
<tr>
<td>Achieve full potential</td>
</tr>
<tr>
<td>Feeling alone</td>
</tr>
<tr>
<td>Developing cancer</td>
</tr>
<tr>
<td>Sexual performance</td>
</tr>
<tr>
<td>Feelings about my body</td>
</tr>
<tr>
<td>Financial difficulties</td>
</tr>
<tr>
<td>Effects of medication</td>
</tr>
<tr>
<td>Attractiveness</td>
</tr>
<tr>
<td>Intimacy</td>
</tr>
<tr>
<td>Producing unpleasant odours</td>
</tr>
<tr>
<td>Feeling dirty or smelly</td>
</tr>
<tr>
<td>Being treated as different</td>
</tr>
<tr>
<td>Passing the disease to others</td>
</tr>
<tr>
<td>Ability to have a child</td>
</tr>
</tbody>
</table>
Table 4.2. Questions of the SBSQoL™ scale.

<table>
<thead>
<tr>
<th>SBSQoL™ Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBSQoL1: How did you feel in general during the past week?</td>
</tr>
<tr>
<td>SBSQoL2: During the past week, how much did your illness interfere with your</td>
</tr>
<tr>
<td>everyday activities?</td>
</tr>
<tr>
<td>SBSQoL3: During the past week, how much did your illness interfere with your</td>
</tr>
<tr>
<td>working life / ability to work?</td>
</tr>
<tr>
<td>SBSQoL4: During the past week, how much did your illness interfere with your</td>
</tr>
<tr>
<td>leisure activities?</td>
</tr>
<tr>
<td>SBSQoL5: During the past week, how much did your illness affect your social</td>
</tr>
<tr>
<td>life?</td>
</tr>
<tr>
<td>SBSQoL6: During the past week, how much did your illness interfere with your</td>
</tr>
<tr>
<td>energy level?</td>
</tr>
<tr>
<td>SBSQoL7: During the past week, how much did your illness affect your physical</td>
</tr>
<tr>
<td>health?</td>
</tr>
<tr>
<td>SBSQoL8: During the past week, how much did your illness affect your mobility</td>
</tr>
<tr>
<td>and self-care activities?</td>
</tr>
<tr>
<td>SBSQoL9: During the past week, how much were you affected by pain resulting</td>
</tr>
<tr>
<td>from your illness?</td>
</tr>
<tr>
<td>SBSQoL10: During the past week, how much did your illness interfere with your</td>
</tr>
<tr>
<td>diet, eating and drinking habits?</td>
</tr>
<tr>
<td>SBSQoL11: During the past week, how much did your illness affect your</td>
</tr>
<tr>
<td>emotional life?</td>
</tr>
<tr>
<td>SBSQoL12: During the past week, how much did your illness affect your sleep?</td>
</tr>
<tr>
<td>SBSQoL13: During the past week, how much were you affected by gastrointestinal</td>
</tr>
<tr>
<td>symptoms resulting from your illness?</td>
</tr>
<tr>
<td>SBSQoL14: During the past week, how much were you affected by fatigue /</td>
</tr>
<tr>
<td>weakness resulting from your illness?</td>
</tr>
<tr>
<td>SBSQoL15: During the past week, how much were you affected by diarrhoea /</td>
</tr>
<tr>
<td>stomal output resulting from your illness?</td>
</tr>
<tr>
<td>SBSQoL16: During the past week, how much were you affected by skeleton /</td>
</tr>
<tr>
<td>muscle symptoms resulting from your illness?</td>
</tr>
<tr>
<td>SBSQoL17: During the past week, how much were you affected by other symptoms /</td>
</tr>
<tr>
<td>discomfort resulting from your illness?</td>
</tr>
</tbody>
</table>
Table 4.3. Pattern and structure matrix for principal component analysis.

<table>
<thead>
<tr>
<th>Items</th>
<th>Pattern coefficients</th>
<th>Structure coefficients</th>
<th>Communalities</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
<td>Component</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1        2    3    4</td>
<td>1        2    3    4</td>
<td></td>
</tr>
<tr>
<td>SBSQoL14</td>
<td>0.978    -0.100 0.052 -0.051</td>
<td>0.961    0.104 0.292 0.109</td>
<td>0.939</td>
</tr>
<tr>
<td>SBSQoL6</td>
<td>0.943    0.117 -0.009 -0.157</td>
<td>0.939    0.303 0.228 0.023</td>
<td>0.916</td>
</tr>
<tr>
<td>SBSQoL12</td>
<td>0.905    0.176 -0.047 -0.054</td>
<td>0.922    0.364 0.174 0.128</td>
<td>0.883</td>
</tr>
<tr>
<td>SBSQoL7</td>
<td>0.854    -0.098 0.282 -0.113</td>
<td>0.882    0.071 0.496 0.011</td>
<td>0.885</td>
</tr>
<tr>
<td>SBSQoL9</td>
<td>0.820    -0.347 0.317 -0.012</td>
<td>0.820    -0.175 0.518 0.077</td>
<td>0.896</td>
</tr>
<tr>
<td>SBSQoL8</td>
<td>0.761    0.004 -0.234 0.089</td>
<td>0.786    0.035 0.517 0.016</td>
<td>0.585</td>
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<tr>
<td>SBSQoL17</td>
<td>0.745    -0.114 0.331 -0.083</td>
<td>0.763    0.256 0.060 0.529</td>
<td>0.752</td>
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<tr>
<td>SBSQoL16</td>
<td>0.700    0.064 -0.084 0.393</td>
<td>0.722    0.178 -0.056 0.239</td>
<td>0.754</td>
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<tr>
<td>SBSQoL11</td>
<td>0.530    0.358 -0.206 0.061</td>
<td>0.705    0.286 0.445 0.543</td>
<td>0.503</td>
</tr>
<tr>
<td>SBSQoL1</td>
<td>0.511    0.131 0.351 0.461</td>
<td>0.612    0.592 -0.099 0.589</td>
<td>0.804</td>
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<tr>
<td>SBSQoL2</td>
<td>0.483    0.441 -0.185 0.446</td>
<td>0.508    0.479 -0.084 0.206</td>
<td>0.838</td>
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<tr>
<td>SBSQoL3</td>
<td>-0.127   0.866 -0.232 -0.019</td>
<td>-0.001   0.839 -0.268 0.065</td>
<td>0.788</td>
</tr>
<tr>
<td>SBSQoL4</td>
<td>-0.054   0.850 0.297 -0.011</td>
<td>0.197    0.835 0.278 0.051</td>
<td>0.781</td>
</tr>
<tr>
<td>SBSQoL5</td>
<td>0.161    0.758 0.316 0.001</td>
<td>0.400    0.791 0.350 0.090</td>
<td>0.775</td>
</tr>
<tr>
<td>SBSQoL13</td>
<td>0.040    -0.009 0.917 0.265</td>
<td>0.306    0.021 0.910 0.214</td>
<td>0.904</td>
</tr>
<tr>
<td>SBSQoL10</td>
<td>0.109    0.335 0.725 -0.252</td>
<td>0.311    0.327 0.765 -0.243</td>
<td>0.759</td>
</tr>
<tr>
<td>SBSQoL15</td>
<td>-0.176   -0.070 0.088 0.086</td>
<td>0.005   -0.005 -0.016 0.942</td>
<td>0.927</td>
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</table>
### Table 4.4. Normality tests.

<table>
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<tr>
<th>Items</th>
<th>Kolmogorov-Smirnov&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shapiro-Wilk</th>
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<tr>
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<td>Statistic</td>
<td>df</td>
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<tr>
<td>SBSQoL2</td>
<td>0.311</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL3</td>
<td>0.302</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL4</td>
<td>0.234</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL5</td>
<td>0.186</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL6</td>
<td>0.355</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL7</td>
<td>0.368</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL8</td>
<td>0.279</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL9</td>
<td>0.233</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL10</td>
<td>0.222</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL11</td>
<td>0.235</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL12</td>
<td>0.234</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL13</td>
<td>0.325</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL14</td>
<td>0.238</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL15</td>
<td>0.221</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL16</td>
<td>0.192</td>
<td>9</td>
</tr>
<tr>
<td>SBSQoL17</td>
<td>0.278</td>
<td>9</td>
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</tbody>
</table>

<sup>a</sup> Lilliefors Significance Correction; <sup>+</sup> This is a lower bound of the true significance.

### Table 4.5. Mann-Whitney U test results.

<table>
<thead>
<tr>
<th>Items</th>
<th>L25%, n=4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>U25%, n=5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mann-Whitney U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBSQoL12</td>
<td>4.0</td>
<td>10.0</td>
<td>3.5</td>
<td>0.095&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBSQoL13</td>
<td>2.0</td>
<td>10.0</td>
<td>3.0</td>
<td>0.059&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBSQoL14</td>
<td>7.5</td>
<td>10.0</td>
<td>0.0</td>
<td>0.012</td>
</tr>
<tr>
<td>SBSQoL15</td>
<td>2.5</td>
<td>10.0</td>
<td>2</td>
<td>0.045</td>
</tr>
<tr>
<td>SBSQoL16</td>
<td>3.5</td>
<td>9.0</td>
<td>2.5</td>
<td>0.063&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Median; <sup>b</sup> Due to a small sample size, these borderline p-values should be interpreted with caution.
4.6 Figures

Figure 4.1. Means of answers to questions SBSQoL1-17.

Figure 4.2. Scree plot.
Figure 4.3. Difference in medians for specific SBSQoL™ questions shown in Table 4.5.
Chapter 5
General Discussion, Conclusion and Future Work

5.1 Summary of New Findings from the Thesis

This chapter will try to summarise the important findings from the whole thesis, then try to provide and connect the findings with existing theories and conceptions and finally provide a guide for future work that may be needed. In the preface, the main questions of the thesis were:

1. Is citrulline being used in clinical practice? If so, then how is it being used (e.g. diagnostically, therapeutically, which particular conditions etc.)?
2. How is citrulline related to the intestine?
3. How can existing knowledge provide grounds to utilize citrulline as a marker to reflect intestinal function (absorption and possibly others)?
4. Can citrulline be a surrogate marker of other consequences related to bowel disease?

Before I answer them, I am going to present a summary of the main findings from the studies in the present thesis. Hence, the summary of new findings of the present thesis is:

1. The first important finding was that citrulline as a term has been used at the end of the 19th century-beginning of the 20th century to describe an extract of the C. colocynthis which was used as a subcutaneous laxative. A comprehensive search was performed which corroborates this finding with extensive old literature mentioning this term. The authors who had described this laxative were German clinical researchers Hiller (1882) and Kohlstock (1892) with multiple citations to their work (see Appendix A). The next new finding was the fact that citrulline (non protein amino acid) was first described as an amino acid by Koga and Ohtake (1914) and not by Wada (1930a) which is the common belief. This was also published in a history of medicine journal (Fragkos and Forbes, 2011a).

2. The second original finding of this thesis focuses on the findings from the second chapter, the systematic review and meta-analysis of human studies on
citrulline and the intestine. This chapter is an extensive review of the literature on citrulline in relation to the intestine (role, function, anatomy) and a statistical analysis of their results. Most importantly, meta-analyses showed that:

a. Citrulline levels are correlated strongly with small bowel length in short bowel syndrome patients (correlation coefficient 0.68).

b. Citrulline is strongly negatively correlated (correlation coefficient -0.56) with intestinal disease severity with regards to enteropathies (coeliac disease, tropical enteropathy, mucositis, and acute rejection in intestinal transplantation; but nor Crohn’s disease).

c. Citrulline cut-off levels have an overall sensitivity and specificity of 80%. There was however variability in the cut-off levels, but the majority of studies used cut-off level of 20 μmol/L.

d. Citrulline levels in untreated coeliac disease patients compared to controls were reduced by 10 μmol/L. Citrulline levels increase with gluten-free diet and with improvement of the enteropathy.

e. Citrulline levels are decreased in critical illness and sepsis. The existing literature indicates that it could possibly be attributed to use of nitric oxide and arginine in sepsis/inflammation leading to a transient decrease in citrulline; similar to the behaviour of albumin as a negative inflammatory marker. Secondly, it could also indicate enteropathy of acute illness.

f. These findings suggest that citrulline be considered a marker of possible acute intestinal injury or intestinal insufficiency.

3. The next finding stems from the clinical study (Chapter 3).

a. The design of the clinical study aims to answer which of the four amino acids citrulline, arginine, glutamine and 3-methylhisitidine and placebo affect post absorptive concentrations of citrulline, glutamine, arginine, ornithine, and 3-methyl-histidine in a more potent manner. The design for this was a fully balanced Williams design crossover study minimizing the effects of carryover or imbalance possibilities.
b. The original findings from the results were that citrulline was the most potent stimulator for all other amino acids, contrary to beliefs of glutamine challenges.

c. There was no effect of age, gender and BMI on the AUC, C_{max} and TOMC. This essentially indicates that basic anthropometric characteristics and age have no effect on pharmacological post-ingestion measures.

d. Citrulline, arginine and ornithine were more affected by the treatment overall (crossover ANOVA p < 0.05, Tables 3.7 and 3.8).

e. There was no carryover and period effect for all treatments, except for 3-methylhistidine which had a significant period effect (possibly due to small concentrations, p < 0.05, Tables 3.7 and 3.8). This confirms that the study design maintained low likelihood for carryover or period effects.

f. Citrulline increased arginine concentrations more significantly compared to glutamine, placebo and 3-methylhistidine but not arginine (p < 0.05 from Tukey multiple comparisons; Table 3.9).

g. Arginine increased arginine concentrations more significantly than glutamine, placebo and 3-methylhistidine (p < 0.05 from Tukey multiple comparisons) but with a less prominent effect than citrulline (Table 3.9, see also Table 3.6, Figure 3.27).

h. Citrulline increased citrulline concentrations more significantly compared to arginine, glutamine, placebo and 3-methylhistidine but not arginine (p < 0.05 from Tukey multiple comparisons, Table 3.9), while arginine increased citrulline significantly less than glutamine only (p < 0.05 from Tukey multiple comparisons, Table 3.9).

i. Citrulline increased ornithine levels significantly compared to arginine, glutamine, placebo and 3-methylhistidine (p < 0.05 from Tukey multiple comparisons, Table 3.9).

j. Arginine also increased ornithine levels compared to glutamine, placebo and 3-methylhistidine (p < 0.05 from Tukey multiple comparisons, Table 3.9) but less prominently than citrulline (p < 0.05 from Tukey multiple comparisons, Table 3.9).
a. No treatment was equivalent to treatment with citrulline for producing equivalent post-absorptive concentrations ($p > 0.05$). With respect to all other concentrations, equivalence was reached with all treatments (Anderson-Hauck test $p < 0.05$).

b. My results raise the interesting prospect that citrulline challenges could be useful in intestinal failure but also in liver failure where urea cycle pathways including glutamine, arginine and ornithine are implicated. Furthermore, gastric emptying and intestinal transit factors were not measured in the present study. This study is among the few studies to trial a challenge test in healthy volunteers with many post-absorptive measurements. Similar studies in the literature include those by Peters et al. (2007a, 2007b); Peters et al. (2007c); Peters et al. (2008b) and Pinto Costa et al. (2013) which have citrulline stimulation tests by glutamine. A different category of studies which investigate citrulline physiology also exists and includes studies by Castillo et al. (1995); Fujita and Yanaga (2007); Rougé et al. (2007); Van De Poll et al. (2007a); Van De Poll et al. (2007b); Thibault et al. (2011).

4. The final study (Chapter 4) was investigation of quality of life in short bowel syndrome patients. This was part of a larger international study. In the UCLH cohort of patients the SBSQoL™ scale is highly reliable in measuring quality of life in patients with short bowel syndrome. Main causes of low quality of life are fatigue, diarrhoea/increased stomal output, lack of sleep, GI symptoms, and muscle pains.

5.2 Limitations

Limitations throughout the thesis and the relevant studies exist. These are:

1. From the meta-analysis, these stem from the increased heterogeneity and possibility of publication bias, detection bias and confounding bias that was documented. It was a pattern in many studies not to analyse confounding factors such as other amino acids, renal function (citrulline’s pathways involve a renal component) and inflammatory state. Also, different measuring methods for citrulline (IEC, HPLC, TMS, ELISA), sample preparation, population parameters, different scales for documenting disease severity, absorption, small
bowl length and so on. Each biological method and clinical assessment scale has inherent variability which is added up when a meta-analysis is performed and this leads to heterogeneity. The random effects models take heterogeneity into account allowing for reliable interpretation of significant associations.

2. From the clinical study, limitations included the open-label design and short duration of follow-up. It was conducted in a small sample of healthy volunteers, who have different bioavailability profiles to patients. Thus, the clinical significance of the findings – namely, challenges in patients for functional reasons – must be clarified in additional studies involving patients with intestinal or liver failure. Additional limitations included the variability in measurements from the subjects which produced increased confidence intervals for the areas under the curves. Renal impairment also plays a significant role when interpreting citrulline plasma values especially in patients – where acute or chronic kidney disease can be invariably present.

3. The SBSQoL™ questionnaire had seventeen items. These need to be correlated with other quality of life questionnaires and explored in larger sample sizes. Also the longitudinal analysis of quality of life needs to be examined. Finally, the present study was not able to differentiate reduced quality of life due to home parenteral nutrition from reduced quality of life due to short bowel syndrome.

5.3 Concluding Remarks, Advice for Practitioners and Future Work

Hence, answering the dissertations general aims and hypotheses, citrulline is currently being used as a marker of intestinal injury in many clinical conditions. Research initially started in animals but since the early 2000s it has expanded to human research quite extensively. Research remains mainly within the field of intestinal failure and associated disciplines and as such measuring citrulline in daily clinical practice has still not been actually realised. Main reasons for this are variability in measurement techniques and non-straightforward interpretation of levels requiring possibly experts in the field.

The intriguing part of citrulline’s metabolism is unique site of production in the intestine. Although intuitively this would indicate that any changes in citrulline would reflect intestinal function, the systematic review from Chapter 2 shows quite
a difference in worldwide practice. Although there was a convergence in practices and results, variability exists. Many studies didn’t analyse confounding factors such as other amino acids, renal function (citrulline’s pathways involve a renal component) and inflammatory state. Hence, for future studies it is suggested that nitric oxide, full amino acid profiles (or at least arginine, glutamine and ornithine) are measured when citrulline is measured, renal function and inflammatory state.

I think an interesting field involves the studies of citrulline in the context of critical illness. Studying patients in intensive care settings has multiple advantages: patients are monitored intensely; there are frequent blood measurements, even multiple times per day; clinical scores are established in the field which indicate severity of organ failure and specifically the gut; and patients are expected to respond eventually or succumb to their illness. Hence, intensive care settings provide a certainty and finality in the clinical settings, that cross-sectional measurements in other clinical settings have proven heterogeneous. Hence, I believe that there is prospect in measuring citrulline in context of critical illness. It will allow clarifying the role of sequential of citrulline measurements and citrulline’s role as a marker of acute intestinal injury. One disadvantage so far obvious are the different study designs amongst studies of citrulline and critical illness.

Chapter 3 essentially demonstrated that citrulline can function successfully as an oral challenge test and better than glutamine. Trying to transfer this knowledge into practice will be challenging but not impossible. Measuring citrulline in clinical practice is difficult because frequent blood sampling in daily practice is always an arduous task. However, as I mentioned before, I think intensive care settings will be more accessible to this type of testing. The usefulness of a challenge test in conditions where these is extensive mucosal enteropathy will be more of use in the short term. These are conditions were patients can undergo intestinal adaptation over a period of time before becoming malnourished. A citrulline challenge test would be possibly useful to assess early-on the extent to which the bowel is compromised so as to intervene sooner.

In Chapter 4, I presented results regarding quality of life in patients with short bowel syndrome. Four factors were extracted from the 17-item scale: general physical symptoms and activities, daily activities, digestive system symptoms, and stoma related symptoms. The main causes of low quality of life were fatigue,
diarrhoea/increased stomal output, lack of sleep, gastrointestinal symptoms, and muscle pains. Due to the transitive property of associations, the findings from this quality of life study suggest that changes of plasma citrulline levels are associated with changes with quality of life in patients with short bowel syndrome, due to the simultaneous association of quality of life and changes in citrulline levels with short bowel syndrome.

The present dissertation investigated various aspects relating to citrulline and the intestine, namely, history, physiology, intestinal disease, and psychosocial effects. Overall, citrulline is currently an important aspect of intestinal pathophysiology. As a marker, citrulline needs to be used more by practitioners who deal with enteropathies (coeliac disease, tropical enteropathy, mucositis, Crohn’s disease and intestinal transplantation) as well as surgical conditions where extensive bowel resection might be needed leading up to short bowel syndrome. Citrulline in these cases has shown to be a marker of severity, small bowel length and need for parenteral nutrition. If one combines this association with the satisfactory sensitivity and specificity of citrulline, this shows that its measurement would benefit practitioners in decision making for therapies and interventions.

Quality of life is decreased in short bowel syndrome patients and this shows the amount of research still needed in short bowel syndrome. The clinical study shows an interesting potential for a citrulline stimulation test with citrulline instead of glutamine in enteropathy and short bowel syndrome patients. Endocrinologists use challenge tests as part of their daily practice to investigate insulin, cortisol or other hormone insufficiencies. The gastroenterology field would benefit from this approach as well with challenge tests for the intestine.

Future work could include:

1. A clinical study with citrulline challenges in enteropathy / short bowel syndrome / mucositis patients.

2. A larger study for quality of life correlating quality of life with other psychosocial parameters and other quality of life scales. Also longitudinal studies to examine responsiveness and other questionnaire related parameters.

3. Studies with sequential measurements of citrulline in the context of critical illness that will correlate citrulline with organ failure severity scores.
4. A topic that the present thesis did not discuss thoroughly was citrulline and cellular studies. There is a good amount of literature investigating the proliferative effects of citrulline on muscle cells. This topic is intriguing in the sense it provides potential for the therapeutic effects in conditions where muscle loss is a problem, such as sarcopenia. Sarcopenia is an issue in elderly patients, cancer patients and also obesity with sarcopenic obesity (Osowska et al., 2004; Osowska et al., 2006; Perez-Guisado and Jakeman, 2010; Boutry et al., 2012; Faure et al., 2012). Future studies in this filed would be interesting in skeletal or smooth muscle cells investigating proliferative and cytotoxic effects of citrulline on these cells.

Hence, in a time when intestinal failure research is growing and intestinal physiology and function still has unresolved mysteries, this thesis’ results could not be more desired. This has been a research endeavour which investigated citrulline in the context of normal physiology with the absorption study, in the context of abnormal conditions with the meta-analysis and the quality of life in short bowel syndrome patients. Results involved aspects of the clinical significance and diagnostic potential of plasma citrulline levels and within the limitations of the present results, new avenues for further research have been suggested.
Appendix A

Bibliography on Citrulline Resin

Table A.1. A bibliographic list of the sources mentioning the term “citrulline” from 1882 to 1930, before the discovery of the modern amino acid citrulline.

<table>
<thead>
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<th>No.</th>
<th>Source</th>
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<tbody>
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<td>Nederlandstijdschriftvoorgeneeskunde. Houten: BohnStafleuvaLoghum; 1894. vol. 38, p. 1010 (Pinkhof, 1894)</td>
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</tbody>
</table>

English Texts

3 Morse WH. New therapeutic agents. Detroit: Davis; 1882. p. 183 (Morse, 1882).
9 Chicago Medical Review. Chicago: Chandler & Engelhard; 1882. vol. 5-6, p. 563 (1882c).
14 Therapeutic Gazette. G. S. Davis; 1883. vol. 7, p. 380.
15 Lescher FH. Recent materia medica: notes on their origin and therapeutics. London: Churchill; 1884. p. 27 (Lescher, 1884).
17 Medical and Surgical Reporter. Philadelphia; 1889. vol. 60, p. 498 (1889b).
21 American Veterinary Review. USA; 1893. vol. 17, p. 138 (Middleton, 1893).
23 Therapeutic Gazette. G. S. Davis; 1893. vol. 17, p. 704 (1893b).
28 Western Druggist. Chicago; 1893. vol. 15, p. 150.
33 The Western Medical Reporter. Chicago; 1893. vol. 15, issue 1, p. 278.
40 The Veterinary Magazine. Philadelphia; 1894. vol. 1, p. 223.

219
<table>
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<td>Wilcox RW. Materia medica and pharmacy. Philadelphia: Blakston; 1905. pp. 543-4</td>
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<td>Croftan AC. Clinical Therapeutics. Chicago: Cleveland Press; 1907. p. 456</td>
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<td>57</td>
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<td>Therapeutic Gazette. Detroit: G. S. Davis; 1910. vol. 34, p. 556</td>
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<td>Prinz H. Dental materia medica and therapeutics: with special reference to the rational application of remedial measures to dental diseases: a textbook for students and practitioners, 5th ed. St. Louis: C.V. Mosby; 1920. p. 612</td>
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<td>Gardner W. Chemical synonyms and trade names: a dictionary and commercial handbook, 3rd ed. C. Lockwood and Son; 1926. p. 89</td>
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**French Texts**

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<td>Journal de Médecine, de Chirurgie et de Pharmacologie. Bruxelles: Lamertain; 1884. vol. 79, p. 174</td>
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<td>Karsten H. Flora von Deutschland, Oesterreich und der Schweiz: Gera-Untermhaus: Köhler; 1895. vol. 2, p. 459</td>
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**Italian Text**

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**Swedish Text**

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Appendix C
Supplementary Materials for Chapter 3 (Pharmacodynamic Study)

C.1 Supplementary Tables

Table C.1. General descriptive statistics regarding mass spectrometry measurements for plasma amino acids.

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Table C.3. Crossover ANOVA for plasma amino acid concentrations (before conversion into AUC).

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*Note for all models: Omnibus measure of separability of treatment and carryover = 77.6393%*
### Table C.4: MANOVA results including age, gender and BMI. Adjusted $R^2 = ^a$ 39.8%; $^b$ 98.0%; $^c$ 0.0%; $^d$ 53.3%; $^e$ 0.0%.

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C.2 Supplementary Figures

**Figure C.1.** Internal standard curves for measuring arginine with LC-MS.

**Figure C.2.** Internal standard curves for measuring citrulline with LC-MS.
Figure C.3. Internal standard curves for measuring glutamine with LC-MS.

Figure C.4. Internal standard curves for measuring ornithine with LC-MS.
Figure C.5. Internal standard curves for measuring 3-methylhistidine with LC-MS.
Figure C.6. Histograms showing distribution of AUC.
Figure C.7. Histograms showing distribution of $C_{\text{max}}$. 
Figure C.8. Scatterplot of citrulline AUC with age by treatment and in total. No differences noted.
Figure C.9. Scatterplot of arginine AUC with age by treatment and in total. No differences noted.
Figure C.10. Scatterplot of glutamine AUC with age by treatment and in total. No differences noted.
Figure C.11. Scatterplot of ornithine AUC with age by treatment and in total. No differences noted.
Figure C.12. Scatterplot of 3-methylhistidine AUC with age by treatment and in total. No differences noted.
Figure C.13. Scatterplot of citrulline $C_{\text{max}}$ with age by treatment and in total. No differences noted.
Figure C.14. Scatterplot of arginine $C_{max}$ with age by treatment and in total. No differences noted.
Figure C.15. Scatterplot of glutamine $C_{\text{max}}$ with age by treatment and in total. No differences noted.
Figure C.16. Scatterplot of ornithine $C_{\text{max}}$ with age by treatment and in total. No differences noted.
Figure C.17. Scatterplot of 3-methylhistidine $C_{\text{max}}$ with age by treatment and in total. No differences noted.
Figure C.18. Scatterplot of citrulline TOMC with age by treatment and in total. No differences noted.
Appendix C: Supplementary Materials for Chapter 3

Figure C.19. Scatterplot of arginine TOMC with age by treatment and in total. No differences noted.
Figure C.20. Scatterplot of glutamine TOMC with age by treatment and in total. No differences noted.
Figure C.21. Scatterplot of ornithine TOMC with age by treatment and in total. No differences noted.
Figure C.22. Scatterplot of 3-methylhistidine TOMC with age by treatment and in total. No differences noted.
Figure C.23. Scatterplot of citrulline AUC with BMI by treatment and in total. No differences noted.
Figure C.24. Scatterplot of arginine AUC with BMI by treatment and in total. No differences noted.
Figure C.25. Scatterplot of glutamine AUC with BMI by treatment and in total. No differences noted.
Figure C.26. Scatterplot of ornithine AUC with age by BMI and in total. No differences noted.
Figure C.27. Scatterplot of 3-methylhistidine AUC with BMI by treatment and in total. No differences noted.
Figure C.28. Scatterplot of citrulline $C_{\text{max}}$ with BMI by treatment and in total. No differences noted.
Figure C.29. Scatterplot of arginine $C_{\text{max}}$ with BMI by treatment and in total. No differences noted.
**Figure C.30.** Scatterplot of glutamine $C_{\text{max}}$ with BMI by treatment and in total. No differences noted.
Figure C.31. Scatterplot of ornithine $C_{\text{max}}$ with BMI by treatment and in total. No differences noted.
Figure C.32. Scatterplot of 3-methylhistidine $C_{\text{max}}$ with BMI by treatment and in total. No differences noted.
Figure C.33. Scatterplot of citrulline TOMC with BMI by treatment and in total. No differences noted.
Figure C.34. Scatterplot of arginine TOMC with BMI by treatment and in total. No differences noted.
Figure C.35. Scatterplot of glutamine TOMC with BMI by treatment and in total. No differences noted.
Figure C.36. Scatterplot of ornithine TOMC with BMI by treatment and in total. No differences noted.
Figure C.37. Scatterplot of 3-methylhistidine TOMC with BMI by treatment and in total. No differences noted.
Figure C.38. Boxplot of AUC for plasma amino acids by treatment and gender. No differences noted.
Figure C.39. Boxplot of $C_{\text{max}}$ for plasma amino acids by treatment and gender. No differences noted.
Figure C.40. Boxplot of TOMC for plasma amino acids by treatment and gender. No differences noted.
Appendix D

Other Important Bibliography on Citrulline

D.1 Other Citrulline Human Studies

These studies are simply cited here with no further explanation. They mainly involve physiological studies of citrulline administration and consequent use by the human body as well as other clinical condition not related to the intestine (Felig et al., 1973; Rajantie et al., 1980a; Rajantie et al., 1980b; Yajima et al., 1982; Largillière et al., 1989; Tuchman, 1989; Castillo et al., 1995; Yu et al., 1995; Castillo et al., 1996; Wu et al., 2004; Mandel et al., 2005; Moinard et al., 2005; Naini et al., 2005; Fujita and Yanaga, 2007; Ligthart-Melis et al., 2007; Rougé et al., 2007; Van De Poll et al., 2007a; Van De Poll et al., 2007b; Wiese et al., 2008; Demacker et al., 2009a; Demacker et al., 2009b; Figueroa et al., 2009; Sureda et al., 2009; Bourdon et al., 2010; Hozyasz et al., 2010; Mao et al., 2010; Perez-Guisado and Jakeman, 2010; Goossens et al., 2011; Shin et al., 2011; Squellerio et al., 2011; Thibault et al., 2011; Bourdon et al., 2012; Wessells et al., 2013; van Wijck et al., 2014; Vidal-Casariego et al., 2014; Blasco-Alonso et al., 2015).

D.2 Reviews, Editorials, Commentaries and Letters to the Editor

There have been quite a few interesting reviews over the years regarding citrulline and metabolism, clinical significance, biochemistry; there have also been interesting reviews about many of the topics touched in the present thesis that might not have been cited but are very useful for a bibliography (Cynober, 1994; Levin, 1994; Nightingale, 1994; Shanbhogue and Molenaar, 1994; Bjarnason et al., 1995; Cynober et al., 1995; Rabier and Kamoun, 1995; Wu, 1998; Füstaff, 2000; Kornberg, 2000; Füstaff, 2001; Goodlad et al., 2001; Mithieux, 2001; Nightingale, 2001; Cynober, 2002; 2003; Alteheld et al., 2003; Boelens and van Leeuwen, 2003; Boirie et al., 2003; Buchman et al., 2003; Calder and Yaqoob, 2003; Cano, 2003; Collin and Vapaaatalo, 2003; Cynober, 2003; Daignault et al., 2003; Darmaun and Cynober, 2003; De Bandt, 2003; Dejong et al., 2003; Dhatariya and Sreekumaran Nair, 2003; Foulks, 2003; Füstaff and Stehle, 2003; Ganapathy et al., 2003; Gleeson and Jeukendrup, 2003; Grimble, 2003; Heyland et al., 2003; Høime Hansen, 2003; Holeček, 2003; Hue and Bertrand, 2003; Kinney, 2003; Leverve, 2003; Mackenzie
and Baracos, 2003; Mahoney and Albina, 2003; Malaisse, 2003; Meijer, 2003; Meijer and Dubbelhuis, 2003; Muscatitoli et al., 2003; Neveux et al., 2003; Nightingale, 2003b; Nightingale, 2003a; Nissim and Welbourne, 2003; Obled et al., 2003; Oehler and Roth, 2003; Patureau Mirand et al., 2003; Radrizzani et al., 2003; Rassin, 2003; Sacks and Kudsk, 2003; Selvaggi et al., 2003; Soeters et al., 2003; vom Dahl and Häussinger, 2003; Wagenmakers, 2003; Wakabayashi, 2003; Waren and Boirie, 2003; Wernerman, 2003; Wu and Morris, 2003; Young and Tharakan, 2003; Abumrad and Barbul, 2004; Brosnan and Brosnan, 2004; Keller et al., 2004; Blijlevens, 2005; Curis et al., 2005; Middleton and Jamieson, 2005; Goulet and Sauvat, 2006; Lin and Stoll, 2006; Nightingale and Woodward, 2006; Romero et al., 2006; Curis et al., 2007; Lutgens and Lambin, 2007; Moinard and Cynober, 2007; Schiller, 2007; Crenn, 2008; Crenn et al., 2008; Cynober et al., 2008; Deutz, 2008; Goulet et al., 2008; Peters et al., 2008a; Lankisch, 2009; Schulzke et al., 2009; Tooley et al., 2009; Wallis et al., 2009; Wu et al., 2009; Crenn and Annane, 2010; Crenn and Cynober, 2010; Cynober et al., 2010; Keur et al., 2010; Oliverius et al., 2010; Crenn et al., 2011; Fragkos and Forbes, 2011b, a; Hurt et al., 2011; Ligthart-Melis and Deutz, 2011; Neu and Walker, 2011; Peters et al., 2011; Peterson and Kerner, 2012; Cynober, 2013; Kaore et al., 2013; Barzal et al., 2014; Kaore and Kaore, 2014; Vaira, 2014; Walker, 2014; Breuillard et al., 2015; Piton and Capellier, 2015).
Appendix E

Stata Code

**This is the analysis strategy for pharmacokinetic data of Chapter 5
*************************************************************************
***1. Analyse amino acids by treatment by doing spaghetti plots for each
amino acid concentration and then by subject.
*** 1. PLOTS .................................................................
***Overall command to produce and save individual graphs
***Treatments 1': Cit==1; Arg==2; Gln==3; Placebo==4; 3MH==5.
forvalues i=1/5 {
clear all
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
***spaghetti plots
***Concentration plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
clear twoway (connected `x'_Concnmolml_1 time if treatment==`i', connect(L))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_con_`x'.png"
***Area Plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
clear twoway (connected `x'_Area_1 time if treatment==`i', connect(L))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_area_`x'.png"
***Mean plot by time
***Concentration plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
meansdplot `x'_Concnmolml_1 time if treatment==`i', ytitle(`x'
Concentration ({&mu}mol/l))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_mcon_`x'.png"
***Area Plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
meansdplot `x'_Area_1 time if treatment==`i', ytitle(`x'
Area)
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_marea_`x'.png"
***plots by subjects
***Concentration plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
clear twoway (connected `x'_Concnmolml_1 time if treatment==`i', connect(L)),
by(id)
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_subjcon_`x'.png"
***Area Plot
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014
analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta",
clear twoway (connected `x'_Area_1 time if treatment==`i', connect(L)), by(id)
graph export "C:\Users\kfragkos\Documents\Citrulline research\September
2014 analysis\'c(current_date)'_tr`i'_subjarea_`x'.png"
}
*************************************************************************
Appendix E: Stata Code

***********************************************************************
****Alternative graph by subjects, where each subject has all five amino acids on its graph
***********************************************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta", clear
twoway (connected Citrulline_Concnmolml_1 time if treatment==1, connect(L) msymbol(O)) ///
(connected Citrulline_Concnmolml_1 time if treatment==2, connect(L) msymbol(D) yaxis(2)) ///
(connected Citrulline_Concnmolml_1 time if treatment==3, connect(L) msymbol(T) yaxis(2)) ///
(connected Citrulline_Concnmolml_1 time if treatment==4, connect(L) msymbol(S) yaxis(2)) ///
(connected Citrulline_Concnmolml_1 time if treatment==5, connect(L) msymbol(Oh) yaxis(2)), ///
by(id, legend(position(6))) ///
legend(title("Amino acid ingested", size(vsmall)) label(1 "Citrulline") label(2 "Arginine") label(3 "Glutamine") label(4 "Placebo") label(5 "3MH") rows(1) size(vsmall))

***********************************************************************
***Overall command to produce and save graphs combine in three rows, one column**************************
***********************************************************************

*****Treatment ==1: Citrulline****************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
twoway (connected `x'_Concnmolml_1 time if treatment==1, connect(L)),
name(`x'_1_1) title("Spaghetti Plot")
meansdplot `x'_Concnmolml_1 time if treatment==1, ytitle("`x' Concentration ({&mu}mol/l)")
name(`x'_1_2) title("Means Plot")
twoway (connected `x'_Concnmolml_1 time if treatment==1, connect(L)), by(id),
title("Concentrations by Subject"))
name(`x'_1_3)
graph combine `x'_1_1 `x'_1_2 `x'_1_3, rows(3) cols(1) ysize(20) xsize(7)
title("`x' Concentrations post ingestion of Citrulline", size(large))
name(tr1_`x')
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\""c(current_date)'_Conc_tr1_`x'.tif", width(1809) height(2800)"
}

***********************************************************************
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH ****************************
***********************************************************************
graph combine tr1_Citrulline tr1_Arginine tr1_Glutamine tr1_Ornithine tr1_A_3MH, rows(1) cols(5) ysize(10) xsize(20)

***********************************************************************

*****Treatment ==2: Arginine*******************************
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
twoway (connected `x'_Concnmolml_1 time if treatment==2, connect(L)),
name(`x'_2_1) title("Spaghetti Plot")
meansdplot `x'_Concnmolml_1 time if treatment==2, ytitle("`x' Concentration ({&mu}mol/l)")
name(`x'_2_2) title("Means Plot")
twoway (connected `x'_Concnmolml_1 time if treatment==2, connect(L)), by(id),
title("Concentrations by Subject"))
name(`x'_2_3)
graph combine `x'_2_1 `x'_2_2 `x'_2_3, rows(3) cols(1) ysize(20) xsize(7)
title("`x' Concentrations post ingestion of Arginine", size(large))
name(tr2_`x')
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\""c(current_date)'_Conc_tr2_`x'.tif", width(1809) height(2800)"
}
Appendix E: Stata Code

***************************
*************
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH  ****************************
*** graph combine tr2_Citrulline tr2_Arginine tr2_Glutamine tr2_Ornithine tr2_A_3MH, rows(1) cols(5) ysize(10) xsize(20)

******Treatment ==3: Glutamine

**********
******Treatment ==4: Placebo

**********
******Treatment ==5: 3MH

clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta", clear
twoway (connected "x’_Concnmolml_1 time if treatment==5, connect(L), name("x’_5_1") title("Spaghetti Plot"))
meansdplot "x’_Concnmolml_1 time if treatment==5, ytitle("x’ Concentration (({&mu}mol/l))") name("x’_5_2") title("Means Plot")
twoway (connected "x’_Concnmolml_1 time if treatment==5, connect(L)), by(id, title("Concentrations by Subject")) name("x’_5_3")
graph combine "x’_5_1 ‘x’_5_2 ‘x’_5_3, rows(3) cols(1) ysize(20) xsize(7) title("x’ Concentrations post ingestion of 3-Methyl-histidine", size(large))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\"c(current_date)'_Conc_tr5_‘x’.tif", width(1809) height(2800)

***************************
*************
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH  ****************************
*** graph combine tr3_Citrulline tr3_Arginine tr3_Glutamine tr3_Ornithine tr3_A_3MH, rows(1) cols(5) ysize(10) xsize(20)

***************************
*************
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH  ****************************
*** graph combine tr4_Citrulline tr4_Arginine tr4_Glutamine tr4_Ornithine tr4_A_3MH, rows(1) cols(5) ysize(10) xsize(20)

***************************
*************
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH  ****************************
*** graph combine tr5_Citrulline tr5_Arginine tr5_Glutamine tr5_Ornithine tr5_A_3MH, rows(1) cols(5) ysize(10) xsize(20)

**********
Appendix E: Stata Code

********** COMBINATION OF ALL GRAPHS INTO ONE GRAPH **********
*** COMBINATION OF ALL GRAPHS INTO ONE GRAPH ****************************
*** graph combine tr5_Citrulline tr5_Arginine tr5_Glutamine tr5_Ornithine
***  tr5_A_3MH, rows(1) cols(5) ysize(10) xsize(20)
***********************************************************************
***********************************************************************
*** histograms and combination of all graphs into one graph
******* AUC and CMAX**********
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
histogram auc_`x'_Concnmolml_1, percent normal name(`x'_auc)
histogram cmax_`x'_Concnmolml_1, percent normal name(`x'_cmax)
}
*************************************************************************
*************************** graphs for age/bmi/gender with auc/cmax/tomc
***************BOXPLOTS OF AUC, CMAX AND TOMC IN ONE GRAPH
clear all
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
graph matrix auc_`x'_Concnmolml_1 Age, by(treatment, total) diagonal(,size(small))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)'_age_auc_`x'.tif", width(2800) height(1809)
}
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
graph matrix auc_`x'_Concnmolml_1 BM, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_BMI_auc_`x’.tif", width(2800) height(1809)
}
*********
******************************
3. Gender / AUC
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph box auc_Arginine_Concnmolml_1 auc_Citrulline_Concnmolml_1 auc_Glutamine_Concnmolml_1 auc_Ornithine_Concnmolml_1 auc_A_3MH_Concnmolml_1, over(treatment) over(Gender) xsize(20) ysize(8)
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_boxplots_gender_auc.tif"
}
************************CMAX*********************************************
**1. Age / CMAX
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix cmax_`x'_Concnmolml_1 Age, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_age_cmax_`x’.tif", width(2800) height(1809)
}
2. BMI / CMAX********************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix cmax_`x'_Concnmolml_1 BMI, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_BMI_cmax_`x’.tif", width(2800) height(1809)
}
3. Gender / CMAX*****************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix cmax_`x'_Concnmolml_1 BMI, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_BMI_cmax_`x’.tif", width(2800) height(1809)
}
1. Age / TOMC**********************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix tomc_`x'_Concnmolml_1 Age, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_age_tomc_`x’.tif", width(2800) height(1809)
}
2. BMI / TOMC********************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix cmax_`x'_Concnmolml_1 BMI, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_BMI_cmax_`x’.tif", width(2800) height(1809)
}
3. Gender / TOMC**********************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix cmax_`x'_Concnmolml_1 BMI, by(treatment, total) diagonal(size(small)) xlabel(,labsize(vsmall)) ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)’_BMI_cmax_`x’.tif", width(2800) height(1809)
}
Appendix E: Stata Code

2. BMI / TOMC

```stata
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    clear all
    use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
    graph matrix tomc_`x'_Concnmolml_1 BMI, by(treatment, total)
    diagonal(,size(small)) xlabel(,labsize(vsmall))
    ylabel(,labsize(vsmall))
    graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)'_BMI_tomc_`x'.tif", width(2800) height(1809)
}
```

3. Gender / TOMC

```stata
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
graph box tomc_Arginine_Concnmolml_1 tomc_Citrulline_Concnmolml_1 tomc_Glutamine_Concnmolml_1 tomc_Ornithine_Concnmolml_1 tomc_A_3MH_Concnmolml_1, over(treatment) over(Gender) xsize(20) ysize(8)
graph export "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\`c(current_date)'_boxplots_gender_tomc.tif"
```

2. PKCROSS: CROSS OVER ANOVA in Concentrations and AREAS

```stata
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\DS_25012015_corrected_nomissingvalues_alltreatments.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
    pkcross `x'_Concnmolml_1, treatment(treatment)
}
```
saveold "$C: \Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_`c(current_date)'.dta"
***********************************************************************
*** SUMMARY STATISTICS **************************************************
clear all
use "$C: \Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
by treatment, sort: summarize auc_* cmax_* tomc_*
***********************************************************************
***********************************************************************
clear all
use "$C: \Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
pkcross auc_`x'_'Concnmolml_1, treatment(treatment)
}
***********************************************************************
*** SUMMARY STATISTICS **************************************************
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
pkcross LN_AUC_`x', treatment(treatment)
pkequiv LN_AUC_`x' treatment period sequence id, compare(1 2) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(1 3) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(1 4) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(1 5) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(2 3) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(2 4) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(2 5) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(3 4) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(3 5) anderson
pkequiv LN_AUC_`x' treatment period sequence id, compare(4 5) anderson
}
Appendix E: Stata Code

*Analysis of reshaped dataset
*Equivalence and effect
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_`c(current_date)'.dta", clear
pkcross auc_Citrulline_Concnmolml_1, treatment(treatment)
pkequiv auc_Citrulline_Concnmolml_1 treatment period period id, compare(1 2)
pkequiv LN_AUC_ARG treatment period sequence id, compare(1 2) anderson
*ANOVA
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_`c(current_date)'.dta", clear
anova auc_Citrulline_Concnmolml_1 id treatment, repeated(treatment)
pcompare treatment, cimargins
********
***************************************************************
****************Miscellaneous stuff******************************
**********************************************************************
clear all
use "C:\Users\kfragkos\Documents\Citrulline research\September 2014 analysis\completedatasetPK_26 Jan 2015.dta", clear
foreach x in Citrulline Arginine Glutamine Ornithine A_3MH {
anova auc_`x'_Concnmolml_1 id treatment, repeated(treatment)
pcompare treatment}
**********************************************************************
**********************************************************************
***Graph of DFBETA for amino acids AUC by ID***************************
****************************************
***citrulline levels
scatter _dfbeta_1 _dfbeta_2 _dfbeta_3 _dfbeta_4 _dfbeta_5 _dfbeta_6 _dfbeta_7 _dfbeta_8 _dfbeta_9 _dfbeta_10 _dfbeta_11 _dfbeta_12 _dfbeta_13 _dfbeta_14 ///
_dfbeta_15 _dfbeta_16 _dfbeta_17 _dfbeta_18 _dfbeta_19 treatment, ylab(-1(.5)3) yline(.20 -.20) xlab(lab,small) title("Plasma Citrulline AUC") ///
legend(pos(2) ring(0) size(tiny) cols(4)) xtitle(,size(small)) name(gcit) msize(tiny small small small small small small small small small small small)
***arginine Levels
scatter _dfbeta_24 _dfbeta_25 _dfbeta_26 _dfbeta_27 _dfbeta_28 _dfbeta_29 _dfbeta_30 _dfbeta_31 _dfbeta_32 _dfbeta_33 _dfbeta_34 _dfbeta_35 _dfbeta_36 ///
_dfbeta_37 _dfbeta_38 _dfbeta_39 _dfbeta_40 _dfbeta_41 _dfbeta_42 treatment, ylab(-1(.5)3) yline(.20 -.20) xlab(lab,small) title("Plasma Arginine AUC") ///
legend(pos(2) ring(0) size(tiny) cols(4)) xtitle(,size(small)) name(garg) msize(tiny small small small small small small small small small small small small)
***Glutamine levels
scatter _dfbeta_47 _dfbeta_48 _dfbeta_49 _dfbeta_50 _dfbeta_51 _dfbeta_52 _dfbeta_53 _dfbeta_54 _dfbeta_55 _dfbeta_56 _dfbeta_57 _dfbeta_58 _dfbeta_59 ///
Appendix E: Stata Code

```stata
*_dfbeta_60 _dfbeta_61 _dfbeta_62 _dfbeta_63 _dfbeta_64 _dfbeta_65
treatment, ylabel(-1(.5)3) yline(.20 -.20) xtitle(val data(small))
title("Plasma Glutamine AUC") ///
legend(pos(2) ring(0) size(tiny) cols(4)) xtitle(size(small)) name(ggln)
msize(small small small small small small small small small small small
small small small small small small small small small)
***Ornithine levels
scatter _dfbeta_70 _dfbeta_71 _dfbeta_72 _dfbeta_73 _dfbeta_74 _dfbeta_75
_dfbeta_76 _dfbeta_77 _dfbeta_78 _dfbeta_79 _dfbeta_80 _dfbeta_81
_dfbeta_82 ///
_dfbeta_83 _dfbeta_84 _dfbeta_85 _dfbeta_86 _dfbeta_87 _dfbeta_88
treatment, ylabel(-1(.5)3) yline(.20 -.20) xtitle(val data(small))
title("Plasma Ornithine AUC") ///
legend(pos(2) ring(0) size(tiny) cols(4)) xtitle(size(small)) name(gorn)
msize(small small small small small small small small small small small
small small small small small small small small small)
***3MH levels
scatter _dfbeta_93 _dfbeta_94 _dfbeta_95 _dfbeta_96 _dfbeta_97 _dfbeta_98
_dfbeta_99 _dfbeta_100 _dfbeta_101 _dfbeta_102 _dfbeta_103 _dfbeta_104
_dfbeta_105 ///
_dfbeta_106 _dfbeta_107 _dfbeta_108 _dfbeta_109 _dfbeta_110 _dfbeta_111
treatment, ylabel(-1(.5)3) yline(.20 -.20) xtitle(val labs(small))
title("Plasma 3-Methyl-histidine AUC") ///
legend(pos(2) ring(0) size(tiny) cols(4)) xtitle(size(small)) name(g3mh)
msize(small small small small small small small small small small small
small small small small small small small small small)
***Combining Graphs
graph combine gcit garg ggln gorn g3mh, xsize(20) ysize(10) title("DFBETAs
by Treatment") imargin(vlarge) altshrink
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