FUNCTIONAL INDICATORS OF FOLATE STATUS IN CATS AND DOGS: USE OF METABOLIC MARKERS TO GUIDE CLINICAL DECISION-MAKING REGARDING FOLATE DEFICIENCY

MARGARET RACHEL COHN-URBACH

A THESIS SUBMITTED IN FULFILMENT OF THE MASTER OF PHILOSOPHY (SCIENCE)

SYDNEY SCHOOL OF VETERINARY SCIENCE

FACULTY OF SCIENCE

THE UNIVERSITY OF SYDNEY

2025

PREFACE

STATEMENT OF ORIGINALITY

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

No content produced by generative AI tools has been used in the preparation of this thesis.

Margaret Cohn-Urbach

As supervisor for the candidature upon which this thesis is based, I can confirm that, to the best of my knowledge, the authorship attribution and intellectual content statements made above are correct.

Supervisor Name: Associate Professor Craig G Ruaux BVSc (Hons I) PhD DACVIM (SAIM)

ABSTRACT

Background: Alterations to folate status are widely recognised in cats and dogs with gastrointestinal disease. Low blood folate concentrations (hypofolataemia) are reported in up to 32% of dogs and up to 40% of cats with chronic enteropathies. The clinical significance of hypofolataemia in small animals, however, remains unclear.

In human medicine, the laboratory finding of hypofolataemia is considered consistent with a diagnosis of folate deficiency, which is treated with folic acid supplementation. Significant efforts have been directed to developing cut-off values of serum folate at which whole-body folate deficiency is most likely to be present in human patients. One method that has been utilised to develop folate cut-off limits is the use of functional indicators to determine the serum folate concentration at which hypofolataemic patients start to display evidence of disruptions to normal folate-dependent biochemical pathways. To date, no functional indicators have been identified in cats and dogs that can accurately predict the presence of folate deficiency.

Urine formiminoglutamic acid (FIGLU) measurement is a well-described functional test for folate deficiency, that has long been used in the field of human medical research. Traditionally a 24-hour urinary excretion test was performed. However, with significant advancements in analytical techniques increasing the sensitivity for detection of metabolites, spot measurement of blood FIGLU has largely taken its place. Blood FIGLU measurement has not previously been investigated as a potential research tool in veterinary medicine. The author proposes that plasma FIGLU measurement shows promise as a metabolic marker of folate deficiency in cats and dogs.

Objectives:

1) To develop and analytically validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative measurement of FIGLU in feline and canine plasma samples.
2) To report ranges of plasma FIGLU in clinically normal cats and dogs.
3) To assess for a correlation between serum folate and plasma FIGLU concentrations in clinically normal cats and dogs.

Methods: An LC-MS/MS method for the quantitative measurement of plasma FIGLU was developed and analytically validated with respect to linearity, accuracy, precision, recovery and dilution integrity. Plasma FIGLU and serum folate (via a chemiluminescence immunoassay) were measured in 11 dogs and 10 cats that were clinically normal. Statistical analysis was performed to assess for correlations between plasma FIGLU and serum folate concentrations in the clinically normal cat and dog groups.

Results: For the measurement of plasma FIGLU, the reported method demonstrated within-run accuracy between 1.89% and 9.02% from the nominal concentrations, between-run accuracy ranging from 1.83% to 11.07% from the nominal concentrations, within-run precision from 2.06% to 6.83%, and between-run precision from 4.19% to 10.86%. The recovery of FIGLU in canine plasma was 92.5%; thus the selected sample preparation method displayed excellent extraction efficiency. Dilution integrity was not demonstrated for FIGLU measurement, however, which appeared to be a result of ion suppression caused by large amounts of endogenous glutamic acid.

Plasma FIGLU concentrations ranged from 4.5 to 21.0 ng/mL in clinically normal dogs, and from 9.1 to 57.0 ng/mL in clinically normal cats. There was no evidence of a correlation between serum folate and plasma FIGLU concentrations in clinically normal cats and dogs.

Conclusions: The described LC-MS/MS method can be concluded as being accurate and precise for the quantitative measurement of canine and feline plasma FIGLU. Findings suggestive of ion suppression were observed, however, and the author recommends that future projects use stable isotope labelled FIGLU as the internal standard in an attempt to minimise the potential detrimental impact of these matrix effects on accuracy of the method.

This study demonstrated that the amounts of FIGLU present endogenously in the plasma of clinically normal cats and dogs is high enough to be quantifiable via LC-MS/MS techniques. Spot plasma FIGLU measurement therefore shows promise as a practical research tool and a potential functional indicator of folate deficiency in cats and dogs.

ACKNOWLEDGEMENTS

I would like to thank my primary supervisor Associate Professor Craig Ruaux for the opportunity of carrying out his excellent project idea, and for his support during the master's project. I would also like to thank my secondary supervisor Professor Merran Govendir for her valuable support and guidance, which have been key to the completion of the writing stage of my thesis.

Many thanks to Dr Atul Bhatnagar, Dr Samiuela Lee and Dr Kang-Yu Peng for all their assistance and advice with the mass spectrometry analysis that was carried out at Sydney Mass Spectrometry, a core research facility at The University of Sydney. This endeavour would not have been possible without Dr Bhatnagar's time, skill and expertise in developing this LC-MS/MS method, in addition to all the training and educational support he provided.

I am extremely grateful to Kirrawee Veterinary Hospital for allowing me to collect samples at their clinic, and I sincerely appreciate all of the staff's help in recruiting animals and their assistance in collecting samples. Also a special thanks to all of the feline and canine study participants and their owners; thank you for sparing a bit of your blood.

I would like to extend my sincere thanks to Christine Black of Veterinary Pathology Diagnostic Services for all her help in providing me with leftover patient samples. I would also like to thank Keith Cheung and Michelle Patpat for their assistance with organising and helping me to navigate the laboratory workspace. I am also grateful to Dr Evelyn Hall for her help in reviewing my statistical analysis. Finally I would like to acknowledge Vetnostics Pathology for performing sample analysis.

This project was supported via start up funding supplied to Associate Professor Craig Ruaux by The University of Sydney School of Veterinary Science, and by an Australian Government Research Training Program (RTP) Scholarship.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ASVCP	American Society of Veterinary Clinical Pathology
BHMT	Betaine-homocysteine methyltransferase
BSA	Bovine serum albumin
BSH	British Society for Haematology
CXP	Cell exit potential
CV	Coefficient of variation
CE	Collision energy
CBC	Complete blood count
DP	Declustering potential
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
D5-Glu	D5-Glutamic acid
ESI	Electrospray ionisation
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FBP	Folate binding protein
FOLH	Folate hydrolase
FRα	Folate receptor alpha
FDA	Food and Drug Administration
FIGLU	Formiminoglutamic acid
FTCD	Formiminotransferase-cyclodeaminase
GI	Gastrointestinal
HQC	High quality control
HILIC	Hydrophilic interaction liquid chromatography
IBD	Inflammatory bowel disease
IS	Internal standard
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LQC	Low quality control
Glu	L-Glutamic acid

MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MMA	Methylmalonic acid
MRP3	Multidrug resistance-associated protein 3
MRM	Multiple reaction monitoring
NCBI	National Center for Biotechnology Information
NRC	National Research Council
PABA	Para-aminobenzoic acid
PBS	Phosphate buffered saline
PCFT	Proton-coupled folate transporter
apABG	*p*-acetamidobenzoylglutamate
pABG	*p*-aminobenzoylglutamate
QC	Quality control
RBC	Red blood cell
RFC	Reduced folate carrier
RNA	Ribonucleic acid
RCPA	Royal College of Pathologists of Australasia
SACD	Subacute combined degeneration
SAMe	S-adenosylmethionine
MS/MS	Tandem mass spectrometry
THF	Tetrahydrofolate
TMS	Trimethoprim-sulfadiazine
UVTHS	University Veterinary Teaching Hospital Sydney
VPDS	Veterinary Pathology Diagnostic Services
WBC	White blood cell
WHO	World Health Organization

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

1.1 FOLATE

1.1.1 Terminology and forms of folate

Folate was first isolated in 1941 from spinach leaves and its name was consequently derived from the Latin word *folium* (leaf).[1] 'Folate', also referred to as vitamin B9, is now a generic term used to describe a family of compounds that display similar chemical structures and nutritional properties.[2] Folic acid is the simplest form of folate to exist and is therefore regarded as the parent compound for this class.[1]

Folic acid does not actually occur in nature, however; it exists only as a synthetic compound.[1] Yet due to its superior stability during food storage and preparation compared to natural folates, folic acid is the form that is preferentially used for vitamin supplements and food fortification.[3] Natural folates have such poor stability that significant losses in activity occur rapidly during storage, over a matter of days to weeks.[4] The biochemical activity of natural folates is further reduced by harvesting, processing and preparation; whereas synthetic folic acid is much more resistant to chemical treatments and remains stable for far longer periods in storage.[4] Consequently the commercial pet food industry is heavily reliant on folic acid fortification, and a major portion of the folates consumed by pets receiving commercial diets would be in the form of folic acid.[5]

Folate is thereby a collective term that includes both synthetic folic acid and naturally occurring folates.[1] Some of the major natural folates include tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MTHF), 10-formyltetrahydrofolate (10-formyl-THF), 5-formyltetrahydrofolate (5-formyl-THF), and 5,10-methylenetetrahydrofolate (5,10-methylene-THF).[6] After ingestion, folates can be detected in serum and plasma in many

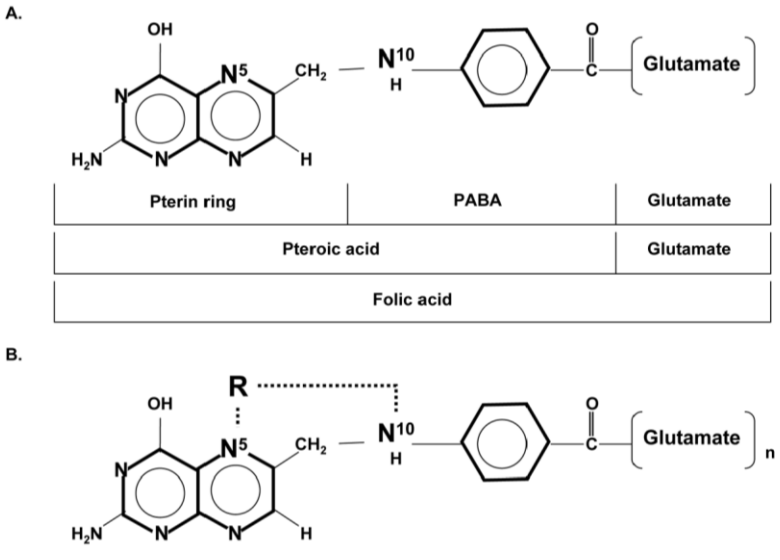
different forms, such as 5-MTHF, 5-formyl-THF, 4-a-hydroxy-5-MTHF, and folic acid.[7] When serum folate is measured in the laboratory, routine immunoassays typically measure a composite blend of all of the different forms of folate.[8] 5-MTHF is the predominant form of folate found in human serum and plasma, however, representing 80 to 90% of total folate in the plasma of healthy humans.[7,9]

To the author's best knowledge, the major forms of folate present in feline and canine blood has not been investigated. 5-MTHF has also been documented to be the main form of folate in the plasma of other animal species such as the rat, however.[10] In one study, 5-MTHF represented over 99% of total folate in murine plasma.[10]

1.1.2 Chemical structure of folate

Folic acid, also called pteroylglutamic acid or pteroyl-L-glutamic acid, is comprised of two major subunits: a pteroyl group and a glutamic acid residue (Figure 1).[1] The pteroyl group is made up of a pteridine (or pterin) ring linked to para-aminobenzoic acid (PABA).[11]

Figure 1. Chemical structures of folic acid (A) and folate (B). Folate compounds differ from each other in terms of the one-carbon substituent (R) at the N5 or N10 position; and the number of glutamic acid residues (n). From page 268 of Kim (2007).[2]



R = CH_3 (N^5), CHO (N^5 & N^{10}), CH=NH (N^5), CH_2 (N^5 & N^{10}) and CH= (N^5 & N^{10})

The pteridine in folic acid is fully oxidised; whereas the pteridine in naturally occurring folates is typically reduced.[2] In addition to differing in the oxidation state of the pteridine, natural folates differ from folic acid in that they: 1) Contain multiple linked glutamic acid residues; and 2) Can contain a one-carbon substitution at the N5 or N10 position (Figure 1).[11] Most naturally occurring folates contain between five and seven glutamate residues.[12] Thus while synthetic folic acid is a 'monoglutamate', most natural folates are 'polyglutamates'.[13] Five different one-carbon substituents have been reported in naturally occurring folates: methyl, formyl, formimino, methylene and methenyl.[12] The chemical structure of folates thereby differ from one another based on the following properties: the number of glutamic acid residues attached to the pteroyl group, the type of carbon substituent, and the state of oxidation of the pteridine.[12]

1.1.3 Food sources of folate

Animals are incapable of synthesising folate; thus it is an essential micronutrient that must be obtained through the diet.[14] Natural folates are most abundant in green leafy vegetables and legumes (beans and peas), but are also present in significant amounts in beef liver, poultry, eggs, dairy products, and fruit.[14,15]

Industrial pet food manufacturers routinely add folic acid to their food products in vitamin premixes.[16] These vitamin premixes are used as a strategy to compensate for vitamin losses that occur during extrusion, processing and storage of pet foods.[5] It is widely accepted that several vitamins are sensitive to the physical and chemical treatments that occur during food processing.[5] It has been shown that between 8 and 30% of folic acid content is destroyed during extrusion of dry canine diets, and an additional 5% lost per month during storage.[5] The precise amount of vitamins lost during the processing of each individual pet food, however, is extremely variable.[15] This is dependent on unique factors like the specific

thermal conditions during processing and particular properties of the total food ration (such as mineral content and pH).[15]

To address this variability, pet food manufacturers usually over-fortify with vitamins to levels that far exceed minimum requirements.[15] Folate toxicity has not been described in small animals (cats and dogs), and no maximum dietary concentrations of folic acid have been proposed by supervisory groups like the National Research Council (NRC).[17] Industrially produced pet foods therefore usually contain copious amounts of folate, making inadequate dietary intake an extremely unlikely cause of folate deficiency in small animals on conventional commercial diets.[18]

As for unconventional commercial pet diets and home-prepared diets, however, concerns surrounding inadequate folate content have been raised. An unpublished master's thesis from the University of Vienna (2014) reported a significantly lower serum folate concentration in 15 cats on commercial vegan diets compared to a control group of 20 conventionally fed cats ($P < 0.001$).[19] Eight of 15 cats (53%) on commercial vegan diets displayed a serum folate concentration below the reference interval, compared to five of 20 cats (25%) on a conventional diet.[19] Furthermore, a 2021 case report described two cats living in the same household that developed hypofolataemia whilst receiving a commercial vegan diet.[16] Pet food analysis of the relevant vegan diet found a folic acid content that was 40% lower than the minimum recommended folic acid level set out by The European Pet Food Industry Federation's nutritional guidelines.[16]

1.1.4 Absorption of folate - based on the human medical literature

Folates are hydrophilic molecules and therefore have minimal capacity to penetrate cell membranes by passive diffusion.[20] Intestinal folate absorption and uptake of folate into systemic tissues therefore relies on carrier-mediated transport systems.[20]

Folate is primarily absorbed in the proximal small intestine of humans via a folate-specific transport carrier, 'proton-coupled folate transporter' (PCFT).[21] PCFT is highly expressed in the proximal jejunum and duodenum, which are thought to be the major sites of folate absorption.[22]

Folates must be in the form of monoglutamates to be transported across the intestinal mucosa; PCFT is highly specific for monoglutamate forms of folate.[21] Existing solely as a monoglutamate, folic acid requires no further breakdown prior to absorption.[13] However for natural folates, which are predominantly polyglutamates, an extra hydrolysis step is required before transit into the enterocyte.[11] 'Intestinal glutamate carboxypeptidase II' which is produced within the brush border membrane of the jejunal mucosa, hydrolyses folate polyglutamates in the gut lumen to folate monoglutamates.[21] Glutamate carboxypeptidase II is also commonly termed 'folate hydrolase' (FOLH) and 'folate hydrolase 1' (FOLH1).[23]

Folate monoglutamates are then transported from the intestinal lumen chiefly by PCFT located in the apical brush border membrane of the jejunum and duodenum.[21] PCFT is highly pH dependent and functions optimally in acidic environments.[21] PCFT operates through a secondary active transport system.[24] It utilises the proton gradient present across the enterocyte's apical cell membrane as an energy source to drive folate uphill and into the enterocyte.[24] This transmembrane proton gradient is maintained by sodium-proton exchangers in the apical membrane.[24] Within the acidic microenvironment of a healthy proximal small intestine, PCFT operates extremely efficiently.[24]

After entry into the enterocyte, most folates are converted into 5-MTHF prior to transport to the basolateral membrane.[25] As for the transport of folate across the enterocyte basolateral membrane, the precise mechanism is yet to be determined.[20] However it appears that export across the basolateral membrane is at least partly mediated by the transporter protein

'multidrug resistance-associated protein 3' (MRP3).[26] After exiting the enterocyte, folate enters the hepatic portal system.[24] Once folate reaches the hepatic sinusoids, it is absorbed across the hepatocyte's basolateral (sinusoidal) membrane.[24] Transit across the sinusoidal membrane appears to be mediated by the transporters PCFT, SLC21A6 and SLC21A8.[24]

Folates that reach the liver can either be: stored; secreted into bile (to return to the duodenum and jejunum, and undergo enterohepatic circulation), or exported into the hepatic vein (to enter systemic circulation).[20] The liver is the body's major storage site for folate, and folate is stored there in the form of polyglutamates.[24] Folate can then be mobilised and delivered into systemic circulation whenever needed to satisfy the requirements of peripheral tissues.[21] Folate polyglutamates are hydrolysed back to their monoglutamate forms prior to export out of the liver.[21] Folate monoglutamates are transported across the sinusoidal membrane (possibly via multidrug resistance-associated proteins and bidirectional transporters), into the hepatic sinusoids where they drain into the hepatic vein and eventually reach systemic circulation.[24]

Folate released from the liver into systemic circulation is principally in the form of 5-MTHF monoglutamates.[27] Therefore any folates in the liver that are still in the form of unmodified folic acid, are converted to 5-MTHF.[25] The majority of folate present in plasma is either freely circulating or weakly bound to low affinity binding proteins.[28,29] Only a very small fraction of plasma folate is bound to high affinity folate binding proteins; estimated at less than 5%.[30] Thus, whereas plasma binding proteins play an important role in the transport and sequestration of other small molecules like vitamin B12, they do not appear to play a significant part in performing such functions in folate homeostasis.[28]

The transporter 'reduced folate carrier' (RFC) plays a vital role in delivering folate from systemic circulation into cells.[20] RFC is expressed ubiquitously in essentially every tissue of

the body and functions optimally at a pH of 7.4.[21] At a physiological pH, RFC acts as either the predominant or sole transport mechanism for uptake of folate into systemic tissues.[21] RFC is an organic phosphate antiporter that harnesses energy from the downhill flow of organic phosphates out of the cell, to drive the uphill transport of folate into cells.[21] This secondary active transport system relies on the synthesis of organic phosphates within cells to facilitate the exchange.[31] The energy expended is through ATP consumption, which is required for the synthesis of organic phosphates.[31]

Once folate monoglutamates are taken up into cells, they are converted back into polyglutamates for storage.[2] Folates are better retained in cells in the polyglutamate form than the monoglutamate form; therefore intracellular folate exists principally as polyglutamates.[2]

1.1.5 Absorption of folate - based on the small animal veterinary literature

Our holistic understanding of folate absorption in small animal medicine is based on human and rodent experimental data. Some studies designed for other purposes have incidentally confirmed specific similarities in folate absorption between small animals and humans. However, there are also many other aspects of folate absorption in small animals that are completely void of experimental data, and require extrapolation from humans and rodents.

The current small animal veterinary literature cites the proximal small intestine as being the primary site of folate absorption in cats and dogs.[32,33,34] Some sources also specify 'jejunum' as being the predominant site of folate absorption,[17,35] and others 'proximal jejunum'.[35] Only one of these publications cites experimental data derived from small animals,[17] implying that the conclusion reached by other authors could be based on extrapolation from humans and rodents, rather than confirmatory experimental data derived from cats and dogs. This is reinforced by a statement made by Edward Hall and Kenneth Simpson in 2000: "There is

clear evidence for ileal cobalamin [vitamin B12] absorption in dogs but the site and mechanism of folate absorption have been extrapolated from other species".[35]

Two canine experimental models have demonstrated that the jejunum plays a role in folate absorption in the dog.[36,37] These experimental models support the theory that the proximal small intestine is likely the primary site of folate absorption in the dog.[36,37] They do not, however, provide sufficient data to determine whether the duodenum is involved, nor whether it is just the proximal jejunum or entire jejunum that take part.[36,37]

Hakim *et al.* (1992) demonstrated that the proximal jejunum is an important site for folate absorption in dogs.[36] In this study, a modified Thiry-Vella loop of the proximal jejunum was surgically constructed in six dogs.[36] This involved an 80 cm length of proximal jejunum being isolated; its vascular, lymphatic and nervous connections with the duodenum and jejunum preserved, and then each end of the jejunal segment made accessible externally through openings in the abdominal wall.[36,38] A solution containing folic acid was infused into the jejunal segment, then the effluent from the jejunal loop collected and analysed.[36]

On average, 46% of folate from the instilled solution was absorbed in fed subjects.[36] This indicated that the 80 cm section of canine proximal jejunum had a significant capacity for folate absorption.[36] While this experimental data suggests that proximal jejunum is involved in folate absorption in dogs, it does not rule out the possibility of other portions of intestine potentially also playing important roles.

Bernstein *et al.* (1972) presented data to suggest that folate absorption occurs in the jejunum, however not in the ileum in dogs.[37] In this experimental model, self-filling intestinal blind loops were surgically constructed in 16 dogs.[37] This is an experimental surgical technique used to create an environment of bacterial overgrowth in the constructed blind loop.[39] The surgical construct produces a peristalsis pattern that keeps the loop persistently filled with

intestinal contents, promoting the development of bacterial overgrowth.[39] Different canine subjects had blind loops of either jejunum or ileum, transposed to different levels of the intestinal tract: at the proximal jejunum or the distal ileum.[37] Since intestinal bacterial overgrowth has been associated with the bacterial synthesis of folate and hyperfolataemia, the goal of this study was to identify the level of intestine at which excess folate created by a blind loop would be absorbed.[37] Folate content of fluid in the blind loops was tested and confirmed to be at extremely high levels.[37]

Markedly elevated serum folate activity was observed in dogs in which the intestinal blind loop was attached at the proximal jejunum, whereas no increase in serum folate activity was observed in dogs with an ileal blind loop attached to the distal ileum.[37] In other words, it appeared that excess folate produced within blind loops was only absorbed when the folate passed through jejunum.[37] Once the excess folate only travelled through ileum and beyond, folate absorption did not occur.[37] The authors concluded that these results were indicative of the canine ileum having no to minimal capacity for folate absorption.[39] Therefore this experimental model provides evidence to suggest that the canine jejunum has a key role in folate absorption, whereas the ileum plays an insignificant part.[37]

The small animal veterinary literature describes intestinal folate absorption in cats and dogs as involving a 'jejunal conjugase' and a 'specific jejunal carrier'.[18] Veterinary texts do not name the particular enzymes and transport proteins involved in folate absorption, however.[18,34]

To date, the specific roles played by FOLH1 and PCFT in intestinal folate absorption in cats and dogs have not been closely evaluated. However, both proteins have been documented in dogs and show strong homology to their human counterparts.[40,41] A 2019 study found that FOLH1, a protein that is also referred to as 'prostate-specific membrane antigen', was

expressed at substantial levels in intestinal epithelium collected from healthy dogs.[41] Furthermore, a 2013 study demonstrated significant homology between canine FOLH1 and human FOLH1.[40] Analysis of the two proteins revealed 91% amino acid homology.[40] Canine FOLH1 also displayed several other characteristics similar to human FOLH1, including similarities in the expression, biosynthesis, processing and localisation of canine FOLH1 within Madin-Darby Canine Kidney cells compared to human FOLH1 in mammalian cells.[40]

Given these experimental findings which confirm that canine FOLH1 is expressed in large amounts in the intestinal epithelium and is very similar to human FOLH1 in structure and physiology, it seems reasonable to hypothesise that FOLH1 likely plays a similar role in folate intestinal absorption in dogs as it does in humans. It therefore appears likely that canine FOLH1 hydrolyses folate polyglutamates in the intestinal lumen of dogs, to facilitate the absorption of folate as monoglutamates.

Moreover, while the specific hydrolytic enzyme responsible for this process has not been proven in dogs, studies have demonstrated that hydrolysis of folate polyglutamates to monoglutamates certainly occurs in dogs prior to intestinal absorption.[42] In a 1970 experimental model, isolated folate polyglutamates were injected into the jejunal lumen of eight dogs and a catheter placed in the portal vein to facilitate repeated blood collection.[42] Folate levels in portal blood significantly rose within 5 minutes of injection, and these folates were found to be present in the monoglutamate form.[42] The authors therefore concluded that folate polyglutamates are hydrolysed to folate monoglutamates during the process of intestinal absorption in the dog.[42]

The folate transporter PCFT has been documented in dogs, and based on its likeness with the human ortholog, has been theorised to play a similar role in canine intestinal folate absorption.[43] Qui *et al.* (2007) demonstrated that PCFT is highly conserved in many

mammalian species, including the dog, both at the level of the gene and protein structure.[43] The canine PCFT protein displayed greater than 80% amino acid homology to the mouse PCFT and rat PCFT; and the rodent PCFT proteins shared 87% amino acid identity to human PCFT.[43] Qui *et al*. concluded that based on the genetic and protein structures of PCFT being highly conserved in various mammalian species and nonmammalian vertebrates, in combination with the function of PCFT being conserved in humans, mice and rats: "this suggests that PCFT is evolutionarily conserved for intestinal folate absorption in mammalian species and likely plays this role in nonmammalian vertebrates as well".[43] In other words, Qui *et al.* proposed that PCFT likely plays a critical role in intestinal folate absorption in several mammalian species including the dog.[43]

Data published on the National Center for Biotechnology Information (NCBI) Protein database (GenPept) additionally reveals that PCFT and RFC have been documented in cats and dogs.[44,45,46,47] However, to the author's knowledge there does not appear to be any specific data published in the literature evaluating the level of homology between these proteins and their human orthologs. A formal sequence alignment analysis would be required for each individual pair of orthologs to come to the most accurate assessment.[43] However, in general RFCs also appear to be conserved amongst various species.[27] For instance, a 64 to 66% amino acid homology has been reported between human RFC and various rodent RFCs.[27]

Further studies would be necessary to clearly define the folate absorption pathway in cats and dogs. However based on the limited data available, it does appear that folate transport bears several key similarities between humans and small animals, and therefore extrapolation from humans appears reasonable.

1.1.6 Catabolism and excretion of folate

The elimination of folate in humans has been researched extensively, whereas minimal data is available specifically pertaining to cats and dogs.[48] Therefore unless otherwise stated, the following information in this section will refer to data sourced from the human medical literature.

Folate elimination occurs predominantly through urine, with smaller amounts being excreted in faeces.[49] While some folate is excreted in urine as intact folate monoglutamates, the majority of urine folate elimination occurs through catabolites of folate.[48] Folates are catabolised in the liver to an inactive degradation product, *p*-aminobenzoylglutamate (pABG).[50,51] After undergoing acetylation to form *p*-acetamidobenzoylglutamate (apABG), apABG is excreted in urine.[50] Although large amounts of folate are secreted into bile, most of this is recycled by intestinal absorption.[49] A 2004 study of human subjects quantified the elimination of folate via these different routes: 56% of folate was eliminated in urine as apABG; 6% eliminated as intact folate monoglutamates in urine; and 38% in the faeces as folate monoglutamates and its oxidation products.[48]

Urinary excretion of intact folates is so low because of an extremely efficient renal tubular reabsorption system.[1] Folates in the blood that enter the kidney unbound to serum proteins are filtered at the glomerulus.[20] However, when physiologic amounts of folate are being consumed, urinary excretion of folates is close to zero due to highly effective renal tubular reabsorption.[20] Folate is primarily reabsorbed in humans by folate receptor alpha (FRα) and RFC in the proximal renal tubules.[52] FRα is expressed in large amounts on the luminal surface of the proximal tubular epithelium, at the brush border membrane.[20] In contrast, RFC is highly expressed at the basolateral membrane of the same proximal tubular cells.[53] Therefore renal reabsorption begins with folate being transported across the apical membrane

by FRα, after which the folate is carried across the basolateral membrane by RFC.[53] The folates thereby enter the peritubular fluid, followed by the peritubular capillaries and eventually re-enter systemic circulation.[53]

The process of folate reabsorption in the kidneys has not been explored in small animals. FRα has been documented in cats and dogs, however.[54,55] Human FRα has not been directly compared against cat FRα and dog FRα in the literature. However, a high degree of amino acid sequence homology has been reported between human FRα and FRα's originating from several different mammalian species, including the rat, pig and cow.[56] In the absence of confirmatory data, one can only speculate that renal conservation of folate may operate in a similar fashion in small animals, as it does in humans.

1.1.7 Intracellular metabolism of folate and folate-mediated one-carbon metabolism

Data specifically relating to intracellular metabolism of folate and folate-mediated one-carbon metabolism in small animals is lacking. The major metabolic pathways arising in folate-mediated one-carbon metabolism, however, are thought to be conserved across most mammalian species.[57] In fact, such a large proportion of the knowledge base relating to folate-mediated one-carbon metabolism arises from rodent models, that reviews in human medicine texts detail that their summaries pertain to "mammalian folate-mediated one-carbon metabolism" rather than being specific to humans.[57] Thus while the following information has been sourced from the human medical literature, it is likely that these basic chemical pathways appear very similar in cats and dogs.

Intracellular folate metabolism is closely interrelated to three important cytoplasmic metabolic pathways: the methionine cycle, purine biosynthesis cycle, and thymidylate cycle.[57] Folate, in its biologically active form THF, activates and carries single carbon groups referred to as 'one-carbon units'.[58,59] These include methyl, formyl, methylene, methenyl and

formimino groups.[58] Acting as a co-factor, THF mediates the transfer of these one-carbon units to specific intermediates in the aforementioned metabolic pathways.[58] These pathways thereby involve a series of metabolic reactions that circulate one-carbon units.[60] Consequently these interlinked metabolic cycles are collectively termed 'one-carbon metabolism'.[60] One-carbon metabolism relies on folate to activate one-carbon units in order to proceed with these metabolic pathways.[59]

In the methionine cycle, 5-MTHF donates a methyl group to enable the remethylation of homocysteine, producing methionine.[61] Methionine synthase catalyses this reaction, which is a cobalamin-dependent enzyme.[1] Methionine is converted to S-adenosylmethionine (SAMe), which is the primary methyl donor of several important reactions; including the methylation of DNA, RNA, histones and proteins.[1] By enabling the regeneration of methionine from homocysteine, folate therefore ensures a constant supply of methyl groups for SAMe-mediated methylation reactions.[58]

In addition to playing a key role in the methionine cycle, this demethylation reaction is also important in enabling folate to assume a form that is reliably retained by the cell.[11] 5-MTHF is a poor substrate for conjugation to polyglutamates, and the methyl group must be removed in order for conjugation to proceed.[11] Since folates must be in a polyglutamate form to be retained by the cell, this demethylation reaction thereby ensures conservation of folates within the cell.[11]

The conversion of folate monoglutamates to polyglutamates is achieved in most eukaryotic organisms by the enzyme folylpolyglutamate synthase.[62] As folates can only be transported across cell membranes in monoglutamate form, this conjugation process blocks their carrier-mediated efflux.[49] While this specific reaction has not been closely evaluated in small

animals, the protein folylpolyglutamate synthase has been documented in both cats and dogs on the NCBI GenPept database.[63,64]

Most folate-dependent reactions take place in the cytosol.[57] However, certain folate-dependent chemical reactions in the mitochondrial compartment are critical in helping to ensure an ongoing supply of molecular substrates for the cytoplasmic pathways.[57] Folate-mediated one-carbon metabolism in the mitochondria is responsible for: a) The synthesis of formate, glycine and formylmethionyl-tRNA; and b) The bidirectional reaction of converting serine to/from glycine.[57] Furthermore, several of these amino acids act as one-carbon unit donors and are thereby essential for cytoplasmic one-carbon metabolism.[57] In this way, mitochondrial and cytoplasmic folate metabolism communicate via exchange of the one-carbon donors formate, glycine and serine.[57]

1.1.8 Function of folate

The critical importance of folate rests within the biologic functions of the molecular end-products of the aforementioned metabolic pathways.[58] In short, folate-dependent reactions play essential roles in nucleotide synthesis, amino acid metabolism, and all metabolic processes that rely on a methylation step.[1] Folate is required for one-carbon reactions that allow the: a) Biosynthesis of purines and the pyrimidine, thymine, to ensure normal DNA synthesis and DNA repair; b) Metabolism of methionine, serine, glycine and histidine; and c) Methylation of phospholipids, neurotransmitters, proteins (including histones), RNA and nucleic acids in DNA.[57] Further, these methylating reactions serve an important regulatory function via the modification of gene stability and gene expression, localisation of proteins, and breakdown of small molecules.[65]

1.2 FOLATE DEFICIENCY

1.2.1 Causes of folate deficiency in humans

The leading cause of folate deficiency in humans is inadequate dietary folate intake.[11] This can occur in association with avoidance of specific foods, eating disorders, low socioeconomic status, alcoholism and inadequately supplemented parenteral nutrition.[11] Other major causes of folate deficiency include intestinal malabsorption, conditions of increased folate turnover, and folate antagonist drugs.[11]

Folate deficiency is a common problem reported in certain malabsorptive conditions: Coeliac disease, tropical sprue, and extensive inflammatory bowel disease (IBD; specifically Crohn's disease).[66] Sporadic reports also exist of regional enteritis, lymphoma of the small intestine, Whipple disease, scleroderma and amyloidosis leading to impaired absorption of folate and subsequent folate deficiency.[67] Hereditary folate malabsorption has also been described, in which patients have a specific defect in the absorption of folate across the intestinal wall due to a loss-of-function mutation in the gene that encodes the folate transporter PCFT.[68]

A multifactorial pathogenesis has been suggested for the development of folate deficiency in IBD.[69] While malabsorption can be a factor in some patients with IBD-related folate deficiency, inadequate dietary folate intake and increased folate requirements also appear to be major contributors.[69] Crohn's disease primarily affects the ileum and colon; whereas the proximal small intestine is often spared.[69] Since proximal small intestine is the chief site for folate absorption, intestinal uptake of folate is likely uninterrupted in many patients with Crohn's disease.[69]

Restrictive eating behaviours and avoidance of specific foods is often undertaken by patients with IBD, due to fear of certain foods causing a relapse of symptoms and intestinal

inflammation.[70] While elimination diets are a commonly utilised tool for initial IBD therapy, when patients undertake independent, unsupervised food avoidance and dietary restriction for extended periods, nutritional deficiencies can occur.[70] Inadequate diet has been suggested to be a severely under-recognised cause of nutritional deficiencies in patients with IBD.[71]

Increased folate utilisation by inflammatory cells is a proposed but unproven mechanism for folate deficiency in patients with IBD.[69] Hoffbrand *et al.* (1968) found that folate malabsorption and inadequate dietary folate did not account for the presence of folate deficiency in all of the patients with Crohn's disease investigated in their study.[72] These authors therefore proposed that increased folate utilisation associated with active inflammatory disease could be an important factor in the development of folate deficiency in some patients with Crohn's disease.[72]

Increased folate requirements have been documented in various conditions with high rates of cell turnover.[73] Increased folate utilisation has been described with inflammatory disorders (rheumatoid arthritis, exfoliative dermatitis), neoplastic diseases (carcinoma, lymphoma), and haematological disorders (haemolytic anaemias, myelofibrosis).[73] Increased folate requirements are also observed in certain physiological states including pregnancy, lactation and prematurity.[73]

Finally, folate deficiency may also arise with the use of certain therapeutic drugs.[11] The following drugs have been associated with reduced folate absorption: antiepileptic drugs (phenytoin, primidone), metformin and oral contraceptives.[4,66] Folate antagonists can additionally induce the clinical signs of folate deficiency by interfering with folate metabolism and thereby impairing the normal intracellular utilisation of folate.[4] Folate antagonists include methotrexate, sulphonamides (e.g. sulfamethoxazole, sulfadiazine), diaminopyrimidines (e.g. trimethoprim), aminopterin and triamterene.[11,74]

1.2.2 Causes of hypofolataemia in cats and dogs

Small intestinal disease is regarded as the foremost cause of hypofolataemia in cats and dogs; so much so that serum folate measurement is considered a suitable laboratory test for assessing small intestinal function.[75,76] Chronic enteropathies, in particular, have a high reported prevalence of hypofolataemia.[77,78] Chronic enteropathies, previously referred to as 'inflammatory bowel disease' in cats and dogs, are defined as "a group of idiopathic disorders characterised by chronic persistent or recurrent gastrointestinal (GI) signs".[79]

Hypofolataemia has also been documented in cats and dogs with an inadequate dietary folate intake,[16,19] and in the following disease states: immune-mediated haemolytic anaemia,[80] gastric carcinoma,[81] and chronic kidney disease.[82] The mechanistic causes of folate deficiency were not closely evaluated in these veterinary studies.[80,81,82] However potential underlying mechanisms include: increased folate utilisation (associated with inflammation, neoplasia and increased red blood cell production) and inadequate dietary folate intake (due to reduced overall food intake associated with inappetence).[80]

Conventional commercial pet foods usually contain large amounts of folate.[18] Thus inadequate dietary intake is a very uncommon cause of folate deficiency in cats and dogs receiving conventional diets.[18] Pets receiving unconventional commercial pet foods, however, may be at higher risk of developing hypofolataemia.[16,19] As previously noted in section 1.1.3, in a 2014 observational study from an unpublished master's thesis, 15 cats on commercial vegan diets displayed a significantly lower serum folate concentration compared to 20 conventionally fed cats ($P < 0.001$).[19]

In a 2021 case report by Fantinati *et al.*, two cats in the same household developed hypofolataemia, anaemia and lethargy five months after being started on a commercial vegan diet, which was proven to be deficient in folic acid.[16] A persistent macrocytic, hypochromic,

non-regenerative anaemia was observed in both cats and folic acid supplementation was initially started as the sole therapy, without any changes to diet. After 30 days of folic acid supplementation, serum folate and all haematologic values had returned to normal limits.[16] Fantinati *et al.* concluded that the unconventional vegan diet was the most likely cause of hypofolataemia and anaemia in these two cohabitating cats.[16]

In a 2020 diet intervention trial of 31 dogs, hypofolataemia developed in four of 17 dogs that were randomly allocated to receive a raw frozen (BARF) commercial dog food.[83] In contrast, hypofolataemia was not documented in any of the 14 dogs that were allocated to receive a conventional commercial dry dog food.[83] In dogs that received the raw commercial diet, the serum folate concentration significantly decreased during the trial ($P = 0.001$), and was significantly lower than dogs fed the conventional commercial diet by the end of the diet intervention trial ($P = 0.001$).[83] These three studies give preliminary evidence to suggest that cats and dogs fed vegan and raw diets might be at increased risk of hypofolataemia.[16,19,83]

The following causes of hypofolataemia have also been reported in small animal veterinary texts and review articles: long-term antibiotic treatment,[18] sulfasalazine and widespread malignancy.[35] However, in the absence of any citations provided in these articles, it is unclear whether these factors have been specifically described in small animals or are simply extrapolated from humans.[18,35]

1.2.3 Folate malabsorption in small intestinal disease

In the small animal veterinary literature, folate malabsorption is regarded as the principal mechanism behind the development of hypofolataemia in animals with small intestinal disease.[75,84] To the author's best knowledge, this appears to be based on extrapolation from human medical literature rather than any small animal experimental studies.[85,86] Specifically,

experimental studies on human patients with Coeliac disease are cited in veterinary articles as the basis for their theories.[85,86]

Upon examination of the veterinary literature, one review article and one book chapter appear to provide the most detailed and best cited hypotheses on the cause of hypofolataemia in small animals with intestinal disease.[85,86] Both references propose that disease of the small intestinal mucosa in cats and dogs leads to loss of folate transport carriers and/or reduced deconjugation activity by hydrolytic enzymes.[85,86] Steiner (2014) theorises that damage to hydrolytic enzymes in the intestinal mucosa of affected cats and dogs could lead to an inability to breakdown polyglutamate forms of folate into the monoglutamate form required for transport across the epithelium.[85] Further, a reduced number of functioning folate transporters could prevent the available folate monoglutamates from being absorbed across the intestinal epithelium and eventually into circulation.[85] Taking into account newer research that has recognised the specific hydrolytic and transporter proteins responsible for folate absorption across the mucosa, one could surmise that the current working theory in small animal medicine is that hypofolataemia arises in small intestinal disease due to impairment of normal FOLH1 and PCFT activity in the intestinal mucosa.

The pathophysiology of folate malabsorption in small animals with intestinal disease is yet to be investigated. Therefore, it is yet to be proven that folate malabsorption actually takes place in cats and dogs with small intestinal disease. However, folate malabsorption has been clearly proven in Coeliac disease in humans, and thus provides a useful model from which one can extrapolate the likely events that take place in small intestinal folate malabsorption in cats and dogs.

Coeliac disease is the most common acquired cause of folate malabsorption in humans.[73] This primarily relates to the distribution of the disease's lesions: Coeliac disease preferentially

affects the proximal small intestine, the major site of folate absorption.[73] Further, Coeliac disease commonly produces severe morphologic and functional changes in the intestinal mucosa, a site where certain intricate chemical processes take place that are critical for folate absorption.[87]

Using various experimental techniques, studies have demonstrated that both impaired hydrolysis of folate polyglutamates and impaired transport of folate monoglutamates are contributing factors to folate malabsorption in Coeliac disease.[88,89,90,91] Halsted *et al.* (1977) showed that there is a reduction in the absorption of folates across the jejunal mucosa in human patients with Coeliac disease, through the use of jejunal perfusion techniques.[90] Multiluminal tubes were placed into the jejunum via fluoroscopic guidance, and carbon-14 isotope labelled folates delivered into the jejunum in an infusion solution.[90] Samples of jejunal fluid were aspirated from ports 30 cm downstream from the infusion site, and the concentration of labelled folates measured in the aspirated fluid.[90] Differences in the concentration of labelled folates in the infusion solution compared to that of the aspirated fluid were used to calculate a 'luminal disappearance' value.[90] The mean luminal disappearance of labelled folates in patients with Coeliac disease was significantly lower than in healthy subjects in the control group ($P < 0.001$).[90] Further, Halsted *et al.* (1978) showed that the 48-hour urinary recovery of labelled folates in these same four patients with Coeliac disease was significantly less than in healthy subjects ($P < 0.01$).[91] These two studies clearly demonstrated that folate malabsorption occurs in Coeliac disease and likely contributes to the development of folate deficiency in patients with this disease.[90,91]

Moreover, Halsted *et al*. (1977) showed that folate malabsorption in Coeliac disease results from both decreased hydrolysis of folate polyglutamates and impaired transport of folate monoglutamates.[90] Hydrolytic products of the carbon-14 labelled polyglutamate folates were detected in the aspirated jejunal fluid during the perfusion experiment.[90] Analysis of the

recovery of these hydrolytic products in patients with Coeliac disease compared to healthy subjects suggested a significantly lower rate of hydrolysis in the diseased subjects ($P < 0.001$).[90] Impaired hydrolysis alone, however, did not appear to account for the degree to which luminal disappearance of folates differed between diseased and healthy subjects.[90] Halsted *et al*. (1977) surmised that based on their calculations, the malabsorption of folates that occurs in Coeliac disease is a combined result of both impaired hydrolysis of folate polyglutamates and reduced transport of folate monoglutamates.[90]

In conclusion, folate malabsorption has not been evaluated specifically in small animals with intestinal disease. However, if one assumes that the small intestinal mucosal damage that arises in Coeliac disease in humans is similar to that which arises in small intestinal diseases in cats and dogs, one can extrapolate the likely mechanisms of malabsorption that occur. Experimental studies have clearly proven that intestinal malabsorption occurs in Coeliac disease, and that it involves both defective hydrolysis of folate polyglutamates and impaired transport of folate monoglutamates across the mucosa.[88,89,90,91] One can therefore extrapolate that the mucosal damage that arises in small intestinal diseases in small animals may result in similar pathophysiologic mechanisms of malabsorption.

1.2.4 Clinical manifestations of folate deficiency in humans

In humans, folate deficiency is well known to have potential haematologic, gastrointestinal and neurologic complications.[4] The most common manifestations observed in acquired folate deficiency include a megaloblastic anaemia, neutropenia, thrombocytopenia, oral ulceration and lethargy.[4,11,92] At a much lower frequency, haemolytic anaemias, thrombotic microangiopathies, and demyelinating neuropathies have also been reported.[4]

At a cellular level, folate deficiency can severely impair DNA synthesis and DNA replication.[4] Therefore cells with a high replication rate are most vulnerable during

deficiencies.[4] This explains the frequent involvement of bone marrow and GI tract in the clinical manifestation of folate deficiency.[4]

The characteristic haematologic complication of folate deficiency is a megaloblastic anaemia.[11] Megaloblastic anaemias are marked by the presence of megaloblastic cells in the bone marrow and macrocytic changes to circulating red blood cells.[93] Megaloblastic erythroid precursors display the following morphologic changes compared to their normal counterparts: increased cell size, coarser nuclear chromatin, and nuclear-cytoplasmic asynchrony (a large nucleus of immature morphology accompanied by cytoplasm with a much higher degree of maturity).[94] Megaloblastic anaemias are also typically characterised by a hypercellular bone marrow.[95] Megaloblasts form as a result of defective DNA synthesis occurring in the rapidly dividing bone marrow cells in folate-deficient states.[67] Large numbers of these defective erythroid precursors are lost through apoptosis prior to being released from the bone marrow.[67] Ineffective erythropoiesis thereby leads to both a reduction in the number of red blood cells entering circulation (anaemia), and the production of abnormal red blood cells.[66] Both of these factors contribute to a reduction in the blood's oxygen-carrying capacity.[66]

In megaloblastic anaemias, the underlying defect in cell division also affects other rapidly dividing bone marrow elements.[96] This can result in a concurrent milder degree of thrombocytopenia and neutropenia.[96] Laboratory analysis of peripheral blood is characterised by a pancytopenia, in addition to macrocytosis (elevated mean corpuscular volume [MCV]) and hypersegmented neutrophils.[94] A low reticulocyte count is typically observed with folate deficiencies (non-regenerative anaemia).[97] Neutrophil hypersegmentation and macrocytosis arise at earlier stages than the development of anaemia; these can therefore serve as earlier indicators of folate deficiency in subclinical states.[93]

The best described gastrointestinal complications associated with folate deficiency are mucosal lesions developing in the oral cavity.[4,11,98] An association has been clearly demonstrated between recurrent oral ulceration (termed aphthous stomatitis) and folate deficiency.[99,100,101] Atrophic glossitis (loss of papillae from the dorsal surface of the tongue) and angular cheilitis (inflammatory lesions of the oral commissures) are also common complications reported in folate-deficient patients.[4,102,103]

Abdominal symptoms have been proposed as being potential complications of folate deficiency.[4,11,96] Diarrhoea, vomiting, nausea, abdominal pain and indigestion are suggested to be possible manifestations of folate deficiency.[4] However, while there is a clear association between folate deficiency and the presence of abdominal complaints, these symptoms often reflect underlying small intestinal disease that has pre-dated the folate deficiency.[96] In the absence of high quality evidence, it is currently debatable as to whether folate deficiency does truly have a causal effect on abdominal symptoms and small intestinal dysfunction.[4] This matter is discussed further in the next section (1.2.5).

Neuropsychiatric and neuropathic complications have been well described in folate deficiency.[104] A strong correlation has been established between folate deficiency and various mental symptoms.[105] In a 1962 case of experimentally induced folate deficiency in a man that underwent self-experimentation, fatigue, irritability, insomnia and memory impairment arose and then resolved rapidly and completely with folic acid supplementation.[106] Furthermore, a 2012 review article reports the following mental symptoms as being frequently observed in patients with folate deficiency: “irritation, sluggishness, loss of memory, lightheadedness, difficulties in concentration”.[4]

An association has also been established between folate deficiency and the neuropsychiatric symptoms of depression, psychosis, dementia and cognitive decline.[104,107] However, a causal

relationship between folate deficiency and these psychiatric illnesses has not been confirmed.[4]

Peripheral neuropathies and subacute combined degeneration of the spinal cord (SACD) are uncommon but well-established complications of folate deficiency.[107] SACD is a potentially life-threatening condition marked by progressive myelin degeneration.[96] In the early stages of SACD, patients experience decreased reflexes, paraesthesia and reduced sense of vibration.[96] If left untreated, patients progress to ataxia, paralysis and ultimately death.[96]

Evidence exists to suggest that folate deficiency may play a role in the development of certain human malignancies.[108] However, results from different studies are inconsistent and therefore a definitive causal link between folate deficiency and neoplasia has not been established.[109] Meta-analyses have demonstrated an association between inadequate folate intake and an increased risk for the development of cancers of the head and neck, oral cavity and pharynx, oesophagus, pancreas and urinary bladder.[109] Contrary to this, however, interventional studies that have investigated the impact of folic acid supplementation on cancer risk have shown conflicting results.[108] In fact, several meta-analyses have found either no significant effect of folic acid supplementation on cancer risk, or a detrimental effect in which cancer risk was increased by supplementation.[109] A 2018 review article concluded that: "The relationship between folate and cancer risk remains uncertain, as studies have demonstrated positive, negative, and neutral associations".[109]

1.2.5 Effects of folate deficiency on the small intestinal mucosa

Experimental studies in rodents and select case series in humans have provided evidence to suggest that folate deficiency may induce structural and functional changes in the small intestine.[110,111,112] In a 1973 animal model by Klipstein *et al.*, folate deficiency was induced in weanling rats either by the administration of both a folate-deficient diet and

succinylsulfathiazole (a sulphonamide), or a folate-deficient diet alone.[110] Historically, early investigators experienced difficulties inducing a clinical state of folate deficiency in rodent animal models using folate-deficient diets alone.[110] Folate antagonists, usually sulphonamides, were therefore commonly used concurrent with folate-deficient diets, in order to induce a more reliable and severe state of clinical folate deficiency in subjects.[110] The control group of this 1973 experimental study also included rats that received succinylsulfathiazole concurrent with folic acid, in order to differentiate between drug adverse effects and the effects of folate deficiency.[110]

In the folate-deficient group of this 1973 experimental study, morphologic changes were present in small intestinal biopsies from 18 of 25 rats.[110] Abnormal small intestinal biopsies demonstrated enlargement of the nuclei of the crypt and villous epithelial cells.[110] Klipstein *et al.* (1973) described these crypt and villous epithelial cell nuclei as demonstrating 'megaloblastic changes'.[110] While the term 'megaloblastic' conventionally refers to characteristic abnormalities observed in erythroid cells in bone marrow, similar reversible morphological abnormalities have been observed in epithelial cells of human patients with megaloblastic anaemias caused by folate deficiency.[113] When epithelial cells display increased cell size, nuclear enlargement, or other cytologic changes that are hallmarks of megaloblastic erythroid cells, these epithelial cells are described as demonstrating 'megaloblastic changes'.[113] Megaloblastic changes have been reported in epithelial cells from various tissues that display particularly high cell turnover: buccal, gastric, small intestinal, cervical and vaginal mucosal cells.[113,114]

In Klipstein *et al.*'s 1973 study of rats, crypt hypertrophy and a decreased mitotic index were also observed on all abnormal intestinal biopsies obtained from folate-deficient subjects.[110] In contrast, no morphological changes were observed on histologic examination of the small intestine of all subjects in the control group.[110] Furthermore, rats that developed a severe

folate deficiency displayed diarrhoea, reduced dietary intake, failure to grow, and a scruffy appearance.[110] Xylose and fat absorption, however, remained normal in nearly all deficient subjects.[110] The authors concluded that induction of folate deficiency in weanling rats resulted in structural changes to the small intestinal wall of most deficient subjects, however functional impairment of the small intestine could not be demonstrated based on xylose and fat absorption testing.[110]

Weaker evidence to support these theories of folate deficiency affecting the small intestines exists in the human medical literature, having been presented only in the form of case reports and case series.[111,112,115] A 1970 case report described a man with chronic alcoholism and folate deficiency that presented with diarrhoea and structural lesions in the small intestinal epithelium.[112] Morphologic alterations identified in small intestinal biopsies included megaloblastic changes to the crypt and villous epithelial cells (increased cell size, increased nuclear size, and irregular distribution of nuclear chromatin) and shortened villi.[112] Both the diarrhoea and all histologic abnormalities on intestinal biopsies resolved after folic acid supplementation was instituted as the sole treatment.[112]

A 1972 case series of 11 males with chronic alcoholism and folate deficiency by Hermos *et al.* reported similar folic acid-responsive structural changes to the small intestine.[115] Eight of the patients displayed histologic abnormalities in the small intestines, and in three patients that showed the most severe folate deficiency-related haematologic changes, particularly striking intestinal structural abnormalities were observed.[115] Patients with severe folate deficiency displayed megaloblastic changes to the crypt and villous epithelial cells (increased cell size, larger rounded nuclei), reduced mitotic rate in crypt cells, and shortened villi.[115] Following folic acid supplementation, repeat small intestinal biopsies from these three subjects showed resolution of all histologic changes.[115]

Interpretation of these results are complicated by the fact that folic acid supplementation was not the only treatment instituted in this study.[115] Cessation of alcohol consumption and initiation of a balanced hospital diet were also undertaken.[115] However, based on the structural intestinal lesions showing a close resemblance to abnormalities observed in patients with severe cobalamin deficiency, Hermos *et al.* (1972) concluded that: "it seems likely that folate deficiency is the cause of both the intestinal epithelial cell and the villous lesions in our study".[115]

A 1977 case series described four infants that presented with nutritional folate deficiency and structural alterations to the small intestinal mucosa.[111] In all four cases, folate deficiency was thought to arise solely due to a nutritionally incomplete diet based on goat's milk, which is known to have negligible amounts of folic acid.[111] Small intestinal biopsies demonstrated megaloblastic changes to epithelial cells of the crypts and villi, with both an increase in overall cell size and larger nuclei.[111] Crypt hypertrophy and villous blunting were also present.[111] Disaccharidase assays showed functional intestinal changes in two of the infants.[111]

Folic acid supplementation was initiated as the sole therapy in the infants.[111] All four infants showed a dramatic clinical response to folic acid therapy, and testing was repeated post-treatment in two infants.[111] In the two infants that underwent post-treatment testing, small intestinal histologic changes had resolved and disaccharidase assay results returned to normal.[111] The authors concluded that nutritional folate deficiency in infants, like the folate deficiency of alcoholism, can result in significant structural changes to the small intestinal mucosa, in addition to functional changes.[111]

Contrary to the aforementioned publications, several other articles exist which demonstrate an absence of structural intestinal wall changes in human patients with clinical folate

deficiency resulting from inadequate dietary intake.[110] Again, the quality of evidence is unfortunately limited by observational design and small case numbers, with these studies ranging from one to seven subjects.[106,116,117,118] Overall in the human medical literature, however, a much larger number of patients with folate deficiency have been documented as having normal morphology of the intestinal mucosa, compared to those with intestinal changes.[115] Therefore, if folate deficiency does induce structural changes to the small intestinal mucosa of humans, this seems to occur in only the minority of patients.

In summary, animal models have indicated that folate deficiency can lead to significant morphologic abnormalities in the small intestinal wall of rodents.[110] However, the nature and severity of this folate deficiency is not directly analogous to the naturally occurring folate deficiency that would arise in humans, cats and dogs.[110,116] Winawer *et al.* (1965) propose that these experimental studies, particularly those utilising folate antagonists (sulphonamides), produce an: "acute total folate deprivation and therefore are not strictly comparable to those induced by nutritional folate deficiency".[116]

The possibility of folate deficiency inducing intestinal wall changes has not been properly explored in cats and dogs.[119] One 1982 paper that evaluated dogs with malabsorptive small intestinal disease raised this topic in the body of their discussion.[119] The article cited two of the aforementioned small observational studies which suggested that nutritional folate deficiency can be associated with intestinal mucosal changes in humans.[119] Batt & Morgan (1982) postulated that folate deficiency might itself contribute to the development of intestinal lesions in dogs with chronic small intestinal disease.[119] Should this be the case, it would have important clinical implications in small animal medicine. It would suggest that folic acid supplementation might actually reduce small intestinal lesions in cats and dogs with chronic enteropathies that have concurrent folate deficiency. Similar effects have been described in cats and dogs with chronic enteropathies treated for cobalamin

deficiency.[120,121,122,123] Several observational studies have presented evidence to suggest that cobalamin supplementation is associated with a reduction in gastrointestinal signs.[120,121,122,123]

1.2.6 Clinical changes associated with naturally occurring hypofolataemia in cats and dogs

To date, only a single publication exists that reports clinical complications from naturally occurring folate deficiency in a small animal. In the previously mentioned case report by Fantinati *et al*. (2021) of two cats fed a vegan diet deficient in folic acid, treatment of hypofolataemia with folic acid supplementation resulted in complete resolution of a macrocytic anaemia.[16]

The report described two cohabitating cats fed a commercial vegan diet which, upon pet food analysis, was shown to contain folic acid at a concentration far below the European Pet Food Industry Federation's minimum recommendations (folic acid content of pet food: 37.24 mg/MJ; folic acid minimum recommendations: 60.50 mg/MJ).[16] Both cats presented with lethargy, hyporexia and weight loss, and blood testing revealed a persistent mild macrocytic, hypochromic, non-regenerative anaemia and hypofolataemia.[16] Cat 1 displayed a haematocrit of 21.8% (reference interval 25 – 45%), MCV of 68.3 fL (40 – 55 fL), mean corpuscular haemoglobin concentration (MCHC) of 28.4 g/dL (30 – 35 g/dL), and serum folate of 8.4 nmol/L (23 – 57 ng/mL); and cat 2 displayed a haematocrit of 22.4%, MCV of 61.5 fL, MCHC of 28.6 g/dL and serum folate of 8.8 nmol/L.[16] Folic acid supplementation was initially instituted in both cats as the sole therapy (0.4 mg folic acid per cat administered by mouth once daily), while they continued on the same vegan diet.[16]

After 30 days of folic acid supplementation, all haematologic parameters had returned to normal limits and serum folate had increased to 40.2 nmol/L in cat 1 and 43.9 ng/mL in cat 2.[16] Both cats displayed a slight improvement in appetite (7% increase in caloric intake), however they demonstrated no weight gain or reduction in lethargy.[16] After three months of

folic acid supplementation alone, the pet owners ultimately accepted recommendations for a diet change.[16] Within four months of introduction of a conventional, nutritionally balanced commercial cat food, all remaining clinical signs resolved, with normalisation of appetite, weight and energy levels.[16]

The authors of this 2021 case report concluded that while clinical signs were likely due to a constellation of different vitamin and nutritional deficiencies, the haematologic changes appeared to be primarily a result of severe folate deficiency.[16] The anaemia, macrocytosis and hypochromia showed a complete response to folic acid supplementation alone.[16] This case report suggests that when severe enough, folate deficiency could potentially cause haematologic complications in cats. This is supported by some experimental studies (detailed in section 1.2.7), in which cats and dogs with experimentally induced folate deficiency consistently demonstrated haematologic complications.[124,125,126]

On the other hand, however, three retrospective studies have failed to document any obvious haematologic effects from hypofolataemia, based on a combined study population of 74 hypofolataemic cats and dogs.[127,128,129] Macrocytosis and anaemia were consistently evaluated, and no association was observed between hypofolataemia and either of these blood changes.[127,128,129]

Of particular interest to the topic of haematologic changes in naturally occurring hypofolataemia in small animals, two published studies were specifically designed to investigate the relationship between cobalamin and folate deficiencies, and red cell changes in dogs.[127,128] Ginoudis *et al*. (2024) retrospectively evaluated 47 dogs with chronic diarrhoea, and tested for correlations between hypofolataemia and multiple red cell parameters.[127] Haematocrit, haemoglobin, MCV, MCHC, mean corpuscular haemoglobin (MCH), red cell distribution width, and reticulocyte count were compared in a group of 21

hypofolataemic dogs (serum folate < 21.1 nmol/L) against a group of 26 normofolataemic dogs (serum folate ≥ 21.1. nmol/L) using Spearman's correlation testing.[127] No correlation was observed between folate status and any of the aforementioned haematologic parameters.[127] Spearman's correlation coefficients were 0.166 for haematocrit, 0.158 for MCV, and -0.004 for MCHC, suggesting negligible to no relationship.[127] Chi-square testing was also performed to examine for a relationship between red cell morphological changes on blood smear examination and folate status; no significant differences were observed in the frequency of any of the tested morphological alterations.[127]

The second article presented a retrospective study that evaluated 114 dogs in which an Idexx Laboratories GI Panel (including serum folate measurement) and a complete blood count (CBC) had been performed on the same day.[128] A Fisher's exact test was performed to evaluate the frequency of anaemia in the hypofolataemic group (serum folate < 10.9 nmol/L) against the normofolataemic group (serum folate ≥ 10.9 nmol/L).[128] This retrospective study found that the prevalence of anaemia in the hypofolataemic group (4/13; 31%) did not significantly differ from that of the normofolataemic group (30/100; 30%; P = 0.99).[128] Therefore evidence of a significant association between hypofolataemia and anaemia was not found in this study population.[128] Furthermore, only a single dog in the study population displayed macrocytosis.[128] Specific details were not provided about this dog's folate status; therefore at most, the prevalence of macrocytosis in this group of hypofolataemic dogs would have been 8%.[128] Macrocytosis therefore did not appear to be a widespread complication affecting dogs with hypofolataemia.[128]

A 2007 retrospective study has additionally provided some data on the prevalence of macrocytosis in hypofolataemic cats.[129] The study entailed a search through a veterinary teaching hospital's database for all feline patients that had undergone folate and cobalamin measurement over a seven year period.[129] Of the included 103 cases, 40 cats were

hypofolataemic.[129] The classification of 'hypofolataemia' was based on the laboratory reference interval from which each feline sample was tested; the lower reference limit at each of the four labs were 22 nmol/L, 19.3 nmol/L, 30.5 nmol/L, and 19.5 nmol/L.[129]

None of these hypofolataemic cats displayed macrocytosis.[129] The mean MCV of hypofolataemic cats was also not significantly different from the mean MCV of cats with normofolataemia and normocobalaminaemia (P = 0.63).[129] While two of the hypofolataemic cats were anaemic, they each had another potential underlying cause for the anaemia: chronic renal failure and myelodysplasia.[129] The authors concluded that macrocytosis was not observed as a haematologic complication of hypofolataemia in cats in their study.[129] However, the authors also conceded that macrocytosis occurs as a late occurrence in human patients with low folate status, thereby suggesting that their hypofolataemic cases may not have had a severe enough degree of folate deficiency for this complication to manifest.[129]

Overall, it does appear to be a reasonable inference that folate deficiency-associated anaemia and haematologic changes are uncommon in cats and dogs.[127,128,129] Some key limitations should be appreciated, however, when drawing conclusions from the data. The sample sizes of hypofolataemic cats and dogs in these studies were small (13 to 40 subjects), and the number of anaemic cats and dogs low (6 to 34 subjects).[127,128,129] Even in human medicine where folate deficiency is a well-documented cause of anaemia, less than 4 to 12% of all cases of anaemia are attributable to folate deficiency,[130,131] and similarly only 4 to 10% of patients with folate deficiency display anaemia.[132,133,134]

The current author performed sample size calculations to estimate the number of hypofolataemic animals that would be required for a study to be adequately powered to detect an association between folate deficiency and anaemia. A figure of 6% was used as the estimated incidence of folate deficiency anaemia in hypofolataemic animal subjects. The 6%

incidence has been translated from studies in elderly human patients, where the prevalence of anaemia in subjects with folate deficiency has ranged from 3.7% to 10.3%.[132,133,134] The mean incidence from these three studies was calculated to be 6%.[132,133,134]

Using an incidence of folate deficiency anaemia of 6%, the current author calculated that a sample size of 328 hypofolataemic subjects would be required to perform a study utilising a Fisher's exact test that yields 80% power.[135] Given that studies to date have included only 40 feline subjects and 13 to 21 canine subjects with hypofolataemia, they are likely not sufficiently powered to detect an association between folate deficiency and blood dyscrasias.[127,128,129] Additionally, in human medicine anaemia is considered an advanced stage haematologic sign of folate deficiency.[66] With small sample sizes of hypofolataemic patients, this could result in inadequate numbers of animals experiencing folate deficiency to a sufficiently severe degree to develop folate deficiency anaemia.

Further, the cut-off limit used for serum folate in these studies could affect the ability to detect associations between hypofolataemia and haematologic parameters. The lower reference limits used for the classification of hypofolataemia in these studies ranged from a serum folate of 10.9 nmol/L up to 30.5 nmol/L.[127,128,129] In the human medical field, a lower reference limit of 7 nmol/L is typically used for serum folate measurement.[136] Further research is warranted in the veterinary field to determine if a lower cut-off limit may be indicated. If the lower reference limits used in these studies were inappropriately high, then this could lead to the false designation of animals with adequate folate status as 'hypofolataemic'. This could impair the ability to detect significant differences between designated hypofolataemic and normofolataemic groups. Thus further evaluation of cut-off values for serum folate in cats and dogs could help in identifying potential haematologic complications of folate deficiency in small animals.

Thus overall, haematologic changes do appear uncommon in hypofolataemic cats and dogs. However given the limited statistical power of studies to date, one could not discount macrocytosis and anaemia as still being potential complications of folate deficiency in small animals. Larger sample sizes of cats and dogs with hypofolataemia, including animals with severe folate deficiency, are required to further investigate the relationship between folate deficiency and haematologic abnormalities in small animals.

1.2.7 Clinical manifestations of experimentally induced folate deficiency in cats and dogs

Three publications have demonstrated clinical changes in small animals with experimentally induced folate deficiency.[124,125,126] First, a case report by Afonsky (1954) described an adult dog that had been placed on an inadvertently folate-deficient diet when acting as a control for a nutritional study.[126] The dog displayed a chronic episode of anaemia, which responded rapidly and dramatically to folic acid supplementation, administered as a single subcutaneous dose.[126] During the anaemic episode, anisocytosis and hypochromia were observed on blood smear examination.[126] Additionally, the MCV decreased from 82 fL to 60 fL and the white blood cell (WBC) count decreased from 10 x 10^9/L to 8.1 x 10^9/L.[126] While both were large changes, the MCV and WBC values remained within the reference interval.[126] Four weeks after the blood changes had completely returned to normal, a second episode of anaemia occurred which again displayed a complete response to folic acid supplementation.[126]

Clinical signs additionally observed during the anaemic episodes included a mild reduction in energy levels and atrophic lesions on the dorsum of the tongue (atrophic glossitis).[126] At the conclusion of the study, 278 days after initiation of the presumed folate-deficient diet, the dog was euthanased.[126] Hypoplasia of the bone marrow and atrophic glossitis were detected on post-mortem examination.[126] The authors concluded that experimentally induced folate

deficiency in dogs could produce a syndrome marked by hypochromic anaemia (with a tendency towards microcytosis), bone marrow hypoplasia, and glossitis.[126]

Second, in a larger laboratory study by da Silva *et al.* (1955) folate deficiency was induced in 29 kittens using a combination of a folate-deficient purified diet and sulphonamides.[125] The kittens were aged between two and three months at the start of the experiment, and received a sulphonamide (either phthalylsulfathiazole or sulfaguanidine) for a mean of 172 days.[125] A control group of nine cats received the same purified diet, however with the addition of folic acid.[125]

The study authors reported that by the end of the experiment, most folate-deficient animals had developed anaemia, leucopenia and weight loss.[125] The specific number of cats that developed these complications, however, was not reported.[125] Macrocytosis was also observed in some of the anaemic animals.[125] After the treatment period, folic acid was administered to nine of the 29 deficient cats, and a leucocyte response was observed six to 10 hours later.[125] An improvement in body weight was also observed six to 10 days after folic acid administration; and an increase in red cell values by 30 days.[125] Cats in the control group, who were fed the same purified diet with the addition of folic acid, did not develop anaemia, leucopenia or weight loss.[125,137]

As part of this study, the authors also attempted to induce clinical folate deficiency in an additional 22 cats by dietary manipulation alone.[125] Interestingly, inadequate dietary folate alone was unable to induce clinical signs of folate deficiency in these 22 kittens after up to 393 days of a folate-deficient purified diet.[125] Plasma/serum folate, red blood cell (RBC) folate and liver folate concentrations were not measured in these subjects, however, which would have evaluated more closely for the presence of folate deficiency.[125] Bone marrow sampling was also not performed.[125] The authors concluded that a clinically detectable state

of folate deficiency was not producible in the cats in this experiment, without the inclusion of sulphonamide drugs.[125]

In humans, it has been shown to take as long as 469 days for a folate-deficient diet to produce macrocytic anaemia in a healthy subject undergoing self-experimentation.[138] This delay is thought to result from large stores of folate in the liver.[138] Host and environmental factors appear to also play a significant role in determining the time taken for the development of folate deficiency. For instance, in another case of self-experimentation, anaemia developed after only 126 days of a folate-deficient diet.[106] It is therefore feasible that the length of time of da Silva *et al.*'s cats on the folate-deficient diet (without sulphonamide administration) may have been insufficient to allow for the complete exhaustion of liver folate stores.

A third laboratory study by Thenen and Rasmussen (1978) evaluated three kittens that received a folate-deficient diet for 154 days.[124] An additional three kittens were used as a control group; both groups were fed a purified diet, which was identical for each group other than the inclusion of folic acid supplementation.[124] All cats were aged between six and eight weeks at the start of the experiment.[124]

RBC folate and plasma folate concentrations, which were measured every two weeks, were considerably lower in cats fed the folate-deficient diet compared to the control group at every timepoint.[124] Bone marrow aspiration, liver folate measurement, and urinary excretion of FIGLU after histidine loading, were performed on all cats at the conclusion of the experiment.[124] Liver folate and urinary FIGLU excretion results were suggestive of cats in the folate-deficient diet group suffering from a whole-body folate deficiency.[124] CBCs were additionally performed every two weeks, however, and no clear differences were observed in RBC count, MCV or WBC count between the two groups, in addition to there being no differences in body weight or weight gain.[124] No obvious clinical signs of folate deficiency or

CBC changes were observed in the three folate-deficient cats at the end of the experiment; namely no anaemia, macrocytosis, neutrophilic hypersegmentation, leucopenia or weight loss.[124]

One repeatable abnormality observed in all folate-deficient cats, however, was the presence of marked abnormalities on bone marrow aspirates.[124] Whereas bone marrow aspirates were normal in control cats, those from the folate-deficient cats demonstrated marked megaloblastic changes to the erythroid precursors.[124] The abnormal bone marrow smears demonstrated the following megaloblastic changes in the red blood cell precursors: nuclear-cytoplasmic asynchrony, clumping of chromatin, nuclear fragmentation, and an increased number of mitotic figures.[124]

Morphologic changes in the bone marrow are an early stage haematologic complication of folate deficiency in humans.[66] If folate deficiency is not corrected in a patient with megaloblastic changes in bone marrow, progression of disease is expected to lead to the more advanced stages of folate deficiency which are detectable upon examination of the peripheral blood.[66] Therefore in the earlier stages of folate deficiency, haematologic effects may only be identifiable upon bone marrow examination; whereas in the later stages of folate deficiency, neutrophilic hypersegmentation, macrocytosis and anaemia may be evident.[66]

It appears likely that cats in the experiment undertaken by Thenen and Rasmussen were at an earlier stage of folate deficiency than cats in da Silva *et al.*'s study.[124,125] If Thenen and Rasmussen's experiment had been extended beyond 154 days, it is possible that folate deficiency might have progressed to the point at which changes would be detectable in the peripheral blood and in body weight.[124] Conversely, if bone marrow sampling had of been performed on cats in da Silva *et al.*'s study that displayed no clinical signs of folate deficiency caused by feeding of a folate-deficient diet alone (without sulphonamide

administration), it is possible that these earlier megaloblastic changes in bone marrow might have been observed.[125]

In summary, two studies of cats with experimentally induced folate deficiency and one case report of cats with naturally occurring folate deficiency, have demonstrated similar haematologic complications as those described in humans.[16,124,125] A macrocytic anaemia and hypercellular bone marrow were observed in folate-deficient cats, which are characteristic features seen in folate deficiency in humans.[16,124,125] These studies suggest that a sufficiently severe state of whole-body folate deficiency can result in clinical changes in cats.[16,124,125]

Weaker evidence, in the form of a single case report based in a laboratory setting, suggests that dogs might also develop clinical manifestations of folate deficiency should the deficiency be extreme enough.[126] While some of the complications appear similar in dogs and humans (atrophic glossitis, anaemia, lethargy), the specific haematologic manifestations might differ.[126] While in humans folate deficiency is characterised by hypercellular bone marrow and macrocytosis, in dogs it may manifest as bone marrow hypoplasia and microcytosis.[95,126] Further studies of cats and dogs with severe folate deficiency are required to better elucidate the potential complications of this vitamin deficiency in small animals.

1.2.8 Distinction between folate deficiency and hypofolataemia in the human medical and veterinary literature

A standardised definition for ‘folate deficiency’ does not exist in the human medical literature. Traditionally, however, folate deficiency is considered present when negative folate balance reaches the stage at which there is a reduction in folate tissue stores.[139] Moreover, the earliest stage of negative folate balance is when there is a low serum folate concentration, but body stores are yet to be depleted.[139] This might arise when there is a transient reduction in folate intake.[139]

The liver contains 50% of total body folate and is the primary site for folate storage in the body.[66,140] A low amount of folate in the liver is therefore considered indicative of folate deficiency.[140] Folate concentration of hepatic tissue collected by liver biopsy is thus considered the gold standard test for defining folate deficiency.[140] Clearly the invasiveness of liver biopsy collection makes it unsuitable for routine clinical testing for folate deficiency.[140] Therefore a variety of blood tests are used as surrogate markers for liver folate stores.[140]

A good correlation has been documented between liver folate concentrations, and both serum and RBC folate concentrations in human patients with and without folate deficiency.[141] Wu *et al*. (1975) showed a significant correlation between hepatic folate and serum folate concentrations in 46 human patients that underwent percutaneous liver biopsies ($r = 0.53$, $P < 0.001$). Lower reference limits for serum/plasma folate and RBC folate have been developed for humans, which aim to predict the cut-off value at which folate deficiency is most likely to be present.[142] Over the years, a variety of different methods have been used to calculate the optimal cut-off value.[142]

In the modern era, serum and plasma folate are considered good estimators of tissue folate status, and accurate predictors of folate deficiency.[98] Published guidelines will therefore describe serum folate cut-off values as being "indicative of folate deficiency" and hypofolataemic patients are typically referred to as being deficient in folate.[98]

As veterinary research is yet to establish the serum folate concentration at which tissue folate depletion occurs, the term 'folate deficiency' is rarely used in studies of hypofolataemic cats and dogs.[32,34,80,128] Current veterinary texts and journal articles mostly use the terms 'hypofolataemia' or 'low/decreased serum folate' to describe cats and dogs with serum folate concentrations below the lower reference limit, without specifying if these patients meet the criteria for folate deficiency.[32,34,80,128] For instance, Stanley *et al.* (2019) in their study

consistently refer to canine subjects with serum folate concentrations below the lower reference limit as 'hypofolatemic', but never define them as having folate deficiency.

This discrepancy between the veterinary and human medical literature results from the small animal medical field being uncertain as to whether serum folate concentrations are adequately representative of tissue folate stores in cats and dogs. Whereas there is a confidence in the human medical field that hypofolataemia accurately predicts low liver folate stores and folate deficiency, this correlation has not been proven in small animals.[98,141]

1.2.9 Diagnosis of folate deficiency in human medicine

The most commonly utilised laboratory assays for the evaluation of folate deficiency in human medicine are the measurement of serum folate, plasma folate, RBC folate and plasma homocysteine.[11] Currently, serum folate and RBC folate are most frequently measured in clinical laboratories by competitive folate binding protein assays using chemiluminescence detection systems.[11,98]

Serum and plasma folate are the most popular first-line tests used to assess folate status in human medicine.[11,66] The Royal College of Pathologists of Australasia (RCPA) cites a reference interval of 7 to 40 nmol/L for serum folate in humans at laboratories that participate in nationally harmonised reference intervals.[136] The most recent national guidelines for the diagnosis of folate deficiency published by the British Society for Haematology (BSH) provided recommendations that "A serum folate level < 7 nmol/L (3 μg/L) is indicative of folate deficiency".[98] Therefore, when there is a clinical suspicion of folate deficiency (based on the presence of known risk factors causing deficiency or the observation of haematologic changes suggestive of megaloblastic anaemia), documentation of a serum folate below the lower reference limit is considered diagnostic for folate deficiency.[98]

Both serum and heparinised plasma samples can be utilised by several immunoassays, and results obtained from the two sample types appear comparable.[143,144] The World Health Organization (WHO 2002) recommended that either serum or heparinised plasma samples "can be used without changes of result" for folate testing.[144] It is more common, however, for serum folate to be routinely measured in the clinical setting.[136] UK guidelines (2014) concluded that in the majority of human patients, serum folate measurement alone appears to provide a sufficiently accurate assessment of body folate status.[98]

Some researchers argue, however, that RBC folate concentrations are a more robust marker of body folate status.[66] RBC folate concentrations reflect long-term folate status as they are indicative of body folate status over the lifetime of the red blood cells.[98] Folate is transported into the red blood cell only during its developmental stages.[145] The concentration of folate that accumulates in the red blood cell is therefore determined at the time of red cell production in the bone marrow.[143] RBC folate levels offer an integrated average for the preceding four month period.[143]

Controversy surrounds the topic of whether serum folate or RBC folate should be the first-line test used for routine screening for folate deficiency.[98] Historically, RBC folate was considered superior to serum folate as a marker of folate status.[146] National guidelines developed for the UK in 1994 suggested that a low RBC folate was a more reliable indicator of tissue folate deficiency than a low serum folate.[147] However, many leading authorities have revised these recommendations, including the RCPA and the BSH.[98,136]

The major advantages of RBC folate over serum folate concentrations are that it is not affected by transient changes in dietary folate intake, and that it theoretically may be better at assessing tissue folate stores.[143] These advantages have not necessarily been reflected in clinical studies, however.[148] Studies that have measured liver, serum and RBC folate

concentrations have shown that serum folate correlated equally well with liver folate as RBC folate.[148] In one key study, both serum and RBC folate demonstrated significant correlations with liver folate; and in fact, the correlation between serum and liver folate (r = 0.53) was documented as being slightly higher than the correlation between RBC and liver folate (r = 0.47).[141] A review by Farrell *et al.* (2013) concluded that results from this study provided no evidence to suggest either marker as being superior for the estimation of tissue folate levels.[146]

A key limitation of RBC folate assays is that that they are less precise than serum folate assays.[98] The analytical performance of RBC folate assays is more susceptible to being compromised by pre-analytical and analytical variables.[98] Furthermore, Farrell *et al.* proposed that these analytical variables may be of such great impact, that serum folate might provide a better indication of folate status than RBC folate.[146] Given that RBC folate measurement is also more expensive, slower and more challenging to perform, these likely contribute to why many authorities consider serum folate a superior first-line test.[98,146]

Guidelines presented by the BSH in 2014 recommended that a low serum folate concentration was indicative of folate deficiency and sufficient for diagnostic screening of folate status in most cases.[98] The current pathology manual published by the RCPA similarly recommends serum folate measurement as their preferred screening test for folate deficiency.[136] The RCPA suggest that RBC folate can be used as a second-line test, in cases where there is a high suspicion of folate deficiency but a serum folate concentration at the low end of the reference interval.[136] The RCPA state, however, that "the result of this [RBC folate] testing does not, in general, add to the clinical diagnosis or therapeutic plan".[136]

Plasma homocysteine is a very sensitive functional marker of folate deficiency in humans.[11] Elevated plasma homocysteine levels arise when there is inadequate folate available in the

cells to perform one of its essential functions: the conversion of homocysteine to methionine.[142] High levels of circulating homocysteine usually reflect a failure in the ability of folate to donate a methyl group to homocysteine to facilitate its remethylation and catabolism.[142] Plasma homocysteine can therefore be considered a biomarker of impaired folate-mediated one-carbon metabolism.[149]

Plasma homocysteine can be used as a second-line or third-line test to assess folate status in cases where serum folate and RBC folate results are inconclusive.[143] Some publications recommend plasma homocysteine testing in individuals with a high clinical suspicion of folate deficiency but serum folate and/or RBC folate concentrations within the reference interval.[143] The WHO define serum folate concentrations at the low end of the reference interval (6.8 to 13.4 nmol/L) as being within a 'possible deficiency' range.[142] Sobczyńska-Malefora & Harrington (2018) recommend that plasma homocysteine testing be performed whenever serum folate concentrations return within this 'possible deficiency' range and conclude that: "Suboptimal folate status should be treated, especially if accompanied by elevated total homocysteine".[143]

Generally, however, the medical literature suggests that plasma homocysteine is not used for the routine testing of folate status in a clinical setting.[98] The clinical utility of plasma homocysteine testing is limited by its stringent sampling and analytical requirements, and by its specificity being diminished in cases of comorbid vitamin deficiencies or concurrent inborn errors in homocysteine metabolism.[98,143] While hyperhomocysteinaemia is most commonly due to folate deficiency, elevated plasma homocysteine can also arise with deficiencies in vitamins B12, B6 and B2.[11]

1.2.10 Diagnosis of folate deficiency in cats and dogs

Serum folate is the only marker of folate status routinely measured in pet cats and

dogs.[34,76,128] Serum folate assays are also the only type of testing for folate status that have been validated in cats and dogs.[150,151] Currently the most commonly utilised systems for folate measurement in small animals are human chemiluminescence immunoassays and other types of human immunoassays.[151] The reference interval for serum folate reported by one of the world's largest veterinary laboratories performing folate assays in small animals, the Gastrointestinal Laboratory at Texas A&M University, is 17.5 – 55.5 nmol/L in dogs and 22 - 49 nmol/L in cats.[152]

RBC folate has only been assessed in client-owned animals in a single veterinary paper.[119] In this study of pet dogs with naturally occurring small intestinal disease, eight of 10 dogs with low serum folate concurrently demonstrated low RBC folate, as assessed against a control population of 20 clinically normal dogs.[119] Experimental laboratory studies have additionally measured RBC folate in cats and dogs.[153,154,155] However, the analytical and diagnostic performance of RBC folate assays in cats and dogs are yet to be evaluated.

1.2.11 Methods for developing cut-off values for serum folate in human medicine

The cut-off value for folate deficiency traditionally used by most clinicians for adult humans is 7 nmol/L, which is based on the serum folate concentration at which anaemia is more likely to occur.[98] The WHO initially devised this cut-off value in 1968; it had been determined that the risk of megaloblastic anaemia considerably increased below this serum folate concentration.[98,142,156] Thus the cut-off value for serum folate that is still conventionally used by many in human medicine, was originally developed utilising the haematological indicator of macrocytic anaemia.[142]

The WHO revised their serum folate cut-off value in 2005, however, based on a new method of cut-off development.[142,157] The revised WHO cut-off value was developed based on the functional indicator of plasma homocysteine.[142,158] Two-phase regression models were

constructed to determine the serum folate concentration at which an 'optimum' plasma homocysteine level was achieved.[158] In other words, the cut-off value for serum folate was derived, below which plasma homocysteine concentrations became elevated.[157] These results form the basis for the serum folate cut-off value currently used by the WHO for the classification of folate deficiency, 10 nmol/L.[142]

1.2.12 Functional indicators of folate status investigated in humans

In the broad sense, there are two major approaches to the laboratory assessment of folate status: one approach being the direct measurement of serum, plasma or RBC folate, and the other being the use of functional indicators.[158] Over the years, various functional indicators of folate status have been proposed and investigated by researchers in human medicine.[149] Blood and urine FIGLU concentrations, urinary FIGLU excretion after a histidine load, deoxyuridine suppression testing, DNA methylation, and uracil misincorporation have all been explored as functional indicators of folate deficiency.[66,149] The terms 'metabolic marker' and 'functional marker' are also used synonymously with 'functional indicator' to describe these types of laboratory tests.[142,143,159] These markers all test for impairments to normal biochemical reactions that occur along one-carbon metabolic pathways.[149] Alterations are expected to arise in these markers when tissue folate levels become insufficient to sustain normal folate-dependent biochemical reactions.[139] As these folate-dependent biochemical pathways are expected to operate satisfactorily as long as tissue folate levels are above a certain threshold, functional indicators of folate status are most valuable for differentiating subjects with folate deficiency from those with adequate folate levels.[149] Individuals with a folate status at the low end of the normal range, however, can usually not be differentiated from others in the normal range, or from those with a high folate status.[139]

In the research setting, functional indicators of folate status have primarily been used for the following purposes: establishing cut-off values for serum folate and RBC folate to define folate deficiency;[142,158] assessing the performance of serum folate and RBC folate as markers of folate deficiency;[146] and developing prevalence estimates of folate deficiency in given populations (including prevalence within particular disease groups).[66,160] In the modern day, the only functional indicator of folate status with widespread use amongst researchers is plasma homocysteine.[149]

Plasma homocysteine is actually also used as a functional indicator of cobalamin status.[161] Cobalamin is required as a cofactor in the reaction where homocysteine is converted to methionine by methionine synthase.[161] Plasma homocysteine is considered a sensitive marker of cobalamin deficiency.[161] In one study that evaluated 424 episodes of cobalamin deficiency that occurred in 406 patients, elevated plasma homocysteine was observed in 96% of episodes of cobalamin deficiency.[162] Methylmalonic acid (MMA) is a more specific marker of cobalamin deficiency, however, and so is the preferred functional indicator for assessment of cobalamin status.[161]

1.2.13 Potential use of homocysteine as a marker of folate status in cats and dogs

Studies to date suggest that blood homocysteine may not be useful in evaluating cats and dogs for folate deficiency, although further research is warranted. Only three studies have explored the relationship between folate and homocysteine concentrations in small animals.[163,164,165] Two of these studies specifically only evaluated dogs of the Greyhound breed.[164,165] Dogs of this breed are actually suspected of having a unique primary defect in the conversion of homocysteine to methionine, however, which leads to Greyhound dogs having a significantly higher median serum homocysteine concentration than dogs of other (non-

sighthound) breeds ($P < 0.0001$).[165] Results from these two studies on Greyhound dogs therefore cannot be directly extrapolated to dogs of other breeds.

One study has evaluated the relationship between serum folate and homocysteine in dogs of non-sighthound breeds.[163] In this retrospective study, statistical analysis was performed to assess for a correlation between serum homocysteine and serum folate concentrations in a group of 29 dogs with immunosuppressant responsive chronic enteropathy.[163] Nine of 29 dogs displayed hypofolataemia, and several of these appeared to demonstrate a severe hypofolataemia, with the mean serum folate concentration of these dogs being 10 nmol/L and the laboratory's lower reference limit being 17.5 nmol/L.[163] In this population of both hypofolataemic and normofolataemic dogs, no association was detected between serum homocysteine and serum folate concentrations ($P = 0.440$).[163] An association was observed between serum homocysteine and serum cobalamin concentrations ($P = 0.002$), however this was only a moderate correlation ($r = -0.54$).[163] Thus results from the one study published in the small animal veterinary literature that assesses this relationship, suggest that homocysteine is unlikely useful as a functional indicator of folate status in dogs with gastrointestinal disease.[163]

To the author's best knowledge, there are currently no published studies evaluating the correlation between homocysteine and folate in cats. There is data, however, assessing the relationship between homocysteine and cobalamin in cats.[120,166] Results from two studies suggest that cobalamin deficiency in cats is not associated with elevated serum homocysteine concentrations.[120,166] The remethylation reaction that requires cobalamin as a cofactor to convert homocysteine to methionine is the same reaction that requires folate as a substrate.[161] Therefore, if the metabolism of homocysteine in cats were reliant on this specific biochemical pathway, then it would be expected that a lack of association between cobalamin deficiency

and homocysteine would also likely indicate a poor correlation between folate deficiency and homocysteine concentrations in this species.

Two studies have documented a lack of association between serum cobalamin concentrations and plasma homocysteine concentrations in groups of severely cobalamin-deficient cats.[120,166] In the first study, there was no significant difference in serum homocysteine concentrations identified in 40 cats with severe cobalamin deficiency (serum cobalamin < 100 ng/L) compared to 24 clinically normal control cats.[166]

In a second study by the same authors, serum homocysteine was measured in severely cobalamin-deficient cats prior to and following cobalamin supplementation.[120] The pre-treatment serum homocysteine concentrations of cobalamin-deficient cats was not significantly different from the post-treatment levels, supporting a lack of association between serum homocysteine and cobalamin status in cats.[120] Results from these two studies suggest that serum homocysteine cannot be used as a functional indicator for cobalamin deficiency in cats.[120,166]

The authors of these two studies concluded that: “fasting serum homocysteine concentration does not appear to be dependent upon cobalamin availability in cats”.[120] The authors proposed that cobalamin-deficient cats might have a higher capacity for metabolising homocysteine through alternative pathways, compared to cobalamin-deficient humans.[166] It was hypothesised that during cobalamin-deficient states, cats might be able to up-regulate the betaine-homocysteine methyltransferase (BHMT) pathway to such a degree that cellular and blood homocysteine concentration are maintained within normal limits.[166] This is an alternative methylation reaction that uses betaine as the methyl donor, in place of folate, and the enzyme BHMT, instead of the cobalamin-dependent enzyme methionine synthase.[167] Just as this alternative pathway is not dependent on cobalamin, it is also independent of folate.[167]

In summary, there is direct evidence suggesting that serum homocysteine is a poor functional indicator of folate status in dogs.[163] Only indirect evidence is available for cats, however based on research into cobalamin deficiency, it also appears likely that homocysteine cannot be used as a functional indicator of folate deficiency in cats.[120,166]

1.2.14 Potential use of substrate product ratios as functional indicators of vitamin status

The performance of functional biomarkers of vitamin B status has been shown to be improved in certain human patient populations by the use of substrate product ratios.[168,169,170] Specifically, Ulvik *et al* (2017) evaluated the ratio of homocysteine:cysteine to determine if this index was a better predictor of overall B-vitamin status than homocysteine concentration alone.[169] Along the transsulfuration pathway of one carbon metabolism, homocysteine is converted into cysteine via a series of vitamin B-dependent enzymatic reactions.[170] Therefore in this metabolic pathway, homocysteine is the substrate and cysteine the product.

Studies have demonstrated that the homocysteine:cysteine ratio offered improved sensitivity and specificity for the estimation of B-vitamin status, compared to homocysteine concentration alone, in populations of patients with colorectal cancer, stable heart disease and healthy controls.[169,170] Ulvik *et al.* theorised that such substrate product ratios provided a better estimation of vitamin status due to the following characteristics: 1) As two metabolites along the same pathway are often similarly influenced by the same confounding factors like renal function, calculating their ratio may improve specificity; and 2) As the concentrations of both substrate and product are dependent on B-vitamins acting as cofactors, the resulting ratio may show an improved sensitivity.[169]

1.2.15 Prevalence of hypofolataemia in cats and dogs with chronic enteropathies

Three published retrospective studies have reported a prevalence of hypofolataemia in dogs with histologically confirmed chronic enteropathies at 14%, 31% and 32%.[77,163,171] Petrelli &

Salavati (2019) additionally presented an abstract at the 2019 ECVIM-CA Congress that reported on a larger group of dogs with chronic enteropathies, of which 30% were hypofolataemic (97/321).[172]

A very large laboratory survey by Dandrieux *et al*. (2013) also obtained prevalence estimates of hypofolataemia in 9960 dogs based on sample submissions to a commercial laboratory.[173] Due to the nature of this study design, however, it is more difficult to characterise the study population. The authors asserted that this sampling population would represent a group of dogs presenting with gastrointestinal signs, many of which were likely suffering specifically from chronic enteropathies.[173] Accepting this study's limitations, one can extrapolate that the prevalence of hypofolataemia in dogs presenting with gastrointestinal signs in which there was a clinical suspicion of increased risk of folate deficiency, was 14% (1400/9960).[173] The lower prevalence estimate obtained in this study may be related to differences in the type of folate assay used and the reference interval applied. In contrast to other studies that utilised a competitive immunoassay with lower reference limits ranging from 17.5 to 21.1 nmol/L, Dandrieux *et al*.'s study used a radioassay with a lower reference limit of 8.0 nmol/L.[163,173]

Studies that have evaluated serum folate in cats with chronic enteropathies have obtained discordant results.[78,174,175] In three retrospective studies, the prevalence of hypofolataemia in cats with histologically confirmed chronic enteropathies were reported at 0%, 6% and 40%.[174,175,78]

In a fourth retrospective study, cats were included if serum folate and cobalamin measurement had been undertaken during their diagnostic investigations at a university teaching hospital.[129] While this study design similarly produces a diverse study population, clinical history was available to better characterise the sample group.[129] The most frequent presenting complaints were gastrointestinal signs (vomiting, diarrhoea, weight loss and

anorexia), and the remainder of cats displayed either signs localising to the abdomen or non-specific signs of illness.[129] The resulting study population of 103 animals comprised 59 cats with confirmed gastrointestinal disease (57%), 28 cats with disease in the liver and/or pancreas (27%), and 16 cats with disease in organ systems outside of the alimentary tract.[129] The authors concluded that the prevalence of hypofolataemia in this group of 'ill cats' was 39%.[129]

Therefore in summary, hypofolataemia has been reported in between 14 and 32% of dogs with chronic enteropathies, and in 14% of dogs presenting with gastrointestinal signs. [77,163,172,173] In cats, hypofolataemia has been reported in between 0 and 40% of patients with chronic enteropathies, and in 39% of ill cats.[78,129,174,175]

Table 1. Summary of articles reporting the prevalence of hypofolataemia in dogs with chronic enteropathies

Article	Number of dogs	Diagnosis for sampled dogs	Prevalence of hypofolataemia	Folate assay	Lower reference limit
Benvenuti *et al.* (2020)[163]	29	Immunosuppressant-responsive enteropathy	9/29 (31%)	Competitive immunoassay (Siemens Immulite 1000)	17.5 nmol/L
Petrelli & Salavati (2019)[172]	321	Chronic enteropathy	97/321 (30%)	Not reported	Not reported
Heilmann *et al.* (2018)[171]	127	Chronic inflammatory enteropathy	17/127 (14%)	Competitive immunoassay (Siemens Immulite 2000)	17.5 nmol/L
Moser *et al.* (2018)[77]	41	Chronic lymphocytic-plasmacytic enteropathy	13/41 (32%)	Competitive immunoassay (Roche Elecsys)	21.1 nmol/L
Dandrieux *et al.* (2013)[173]	9960	Dogs presenting with gastrointestinal signs	1400/9960 (14%)	Radioassay (Becton Dickinson & Co, SimulTRAC)[150]	8.0 nmol/L

Table 2. Summary of articles reporting the prevalence of hypofolataemia in cats with chronic enteropathies

Article	Number of cats	Diagnosis for sampled cats	Prevalence of hypofolataemia	Folate assay	Lower reference limit
Kathrani *et al.* (2017)[174]	28	Chronic inflammatory enteropathy	0/28 (0%)	Not reported	19 nmol/L
Burke *et al.* (2012)[175]	18	Chronic gastrointestinal disease (IBD or neoplasia)	1/18 (6%)	Competitive immunoassay (Siemens Immulite 2000)	22 nmol/L
Evans *et al.* (2006)[78]	10	IBD	4/10 (40%)	Not reported	Not reported
Reed *et al.* (2007)	103	'Ill cats'; most presenting with gastrointestinal signs	40/103 (39%)	Competitive immunoassays or radioassay	22 nmol/L, 19.3 nmol/L and 30.5 nmol/L

1.2.16 Therapy for folate deficiency – risks, benefits, and current recommendations

To date, the clinical effect of folic acid supplementation on cats and dogs with gastrointestinal disease has not been assessed.[76] A clinical benefit of folic acid supplementation on hypofolataemic patients with gastrointestinal disease has been neither proven nor disproven in the veterinary literature.[76]

A study by Simpson *et al.* (2023) demonstrated that oral folic acid supplementation was able to normalise serum folate concentrations in hypofolataemic dogs with chronic enteropathies.[176] In six dogs with chronic enteropathies and low serum folate concentrations (median: 6.45 nmol/L; range: 5.05 - 8.95 nmol/L [laboratory reference interval 11.4 – 45.5 nmol/L]), folic acid administration at > 15 μg/kg by mouth for six weeks was associated with normalisation of serum folate in all six dogs (median: 20.8 nmol/L, range: 17.73 – 45.0 nmol/L at six weeks).[176] The clinical effect of this increase in serum folate concentrations, however, was not evaluated or reported by the authors.[176] Additionally, it was not documented whether dogs displayed any clinical complications associated with the

hypofolataemia.[176] Thus, while oral folic acid supplementation was shown to normalise serum folate concentrations, a clinical benefit of this was not confirmed.[176]

There is no consensus in the veterinary community regarding the decisions of whether to and when to treat hypofolataemia in canine and feline patients with gastrointestinal disease. Pundits in the area of veterinary gastroenterology have published the following recommendations: "Oral folic acid supplementation (10 µg/kg or 200-400 µg/dog PO q24h for 30 days) is recommended in patients with moderate or marked hypofolatemia".[177] In the absence of any published evidence defining moderate or marked hypofolataemia, however, one must still use their own judgement in determining cut-off values for when folic acid supplementation is warranted.

A wide range of folic acid doses are also cited in veterinary references, from 200 µg up to 5 mg per cat or dog once daily.[76,178] There is currently insufficient data in the veterinary literature to determine which is the most appropriate dose for cats and dogs.[178]

A key argument asserted by those clinicians that support folic acid supplementation of veterinary patients with gastrointestinal disease, is the extremely favourable safety profile of folic acid therapy.[76] At this point in time, no adverse effects have been reported in cats and dogs receiving oral folic acid supplementation.[17,178]

In the human medical field, however, concerns have been raised that in patients with existing neoplasms, supplementation with very high doses of folic acid may accelerate tumour progression.[179] Supportive evidence of this theory has been obtained in rodent models.[179,180] Randomised control trials of human subjects have produced conflicting results, whereas a meta-analysis did not find evidence of an increased cancer risk associated with folic acid supplementation.[180,181] A working group created by the European Food Safety Authority (EFSA) to assess the risks and benefits of folic acid fortification concluded that: "The current

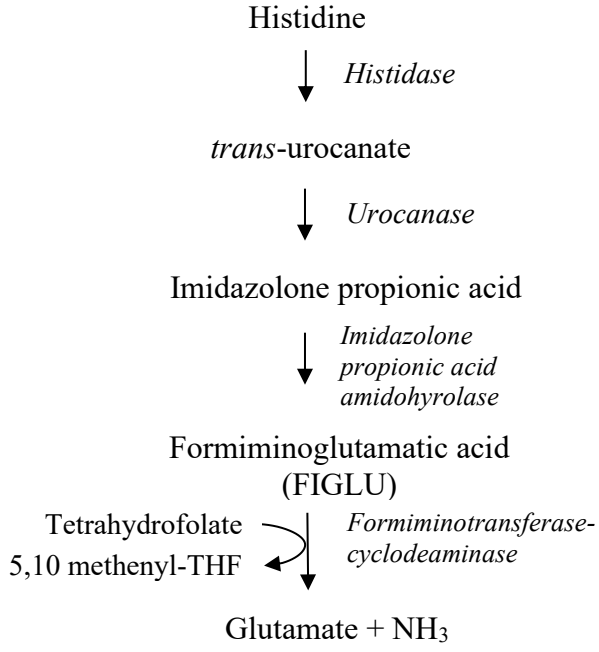
evidence does not show an association between high folic acid intakes and cancer risk but neither do they confidently exclude a risk".[180]

1.3 FORMIMINOGLUTAMIC ACID (FIGLU)

1.3.1 Generation of FIGLU from histidine catabolism – based on the human medical literature

FIGLU is an organic acid metabolite.[182] It is an intermediate metabolite in the catabolism of histidine, an essential amino acid.[183] Histidine catabolism commences with its deamination to *trans*-urocanate and ammonia in a reaction catalysed by histidase, an enzyme that is primarily found in liver and skin (Figure 2).[184] In the liver, urocanase catalyses the hydrolysis of *trans*-urocanate to imidazolone propionic acid, which is then converted to FIGLU.[185]

Figure 2. Catabolism of histidine. Adapted from Brosnan & Brosnan (2020).[184]

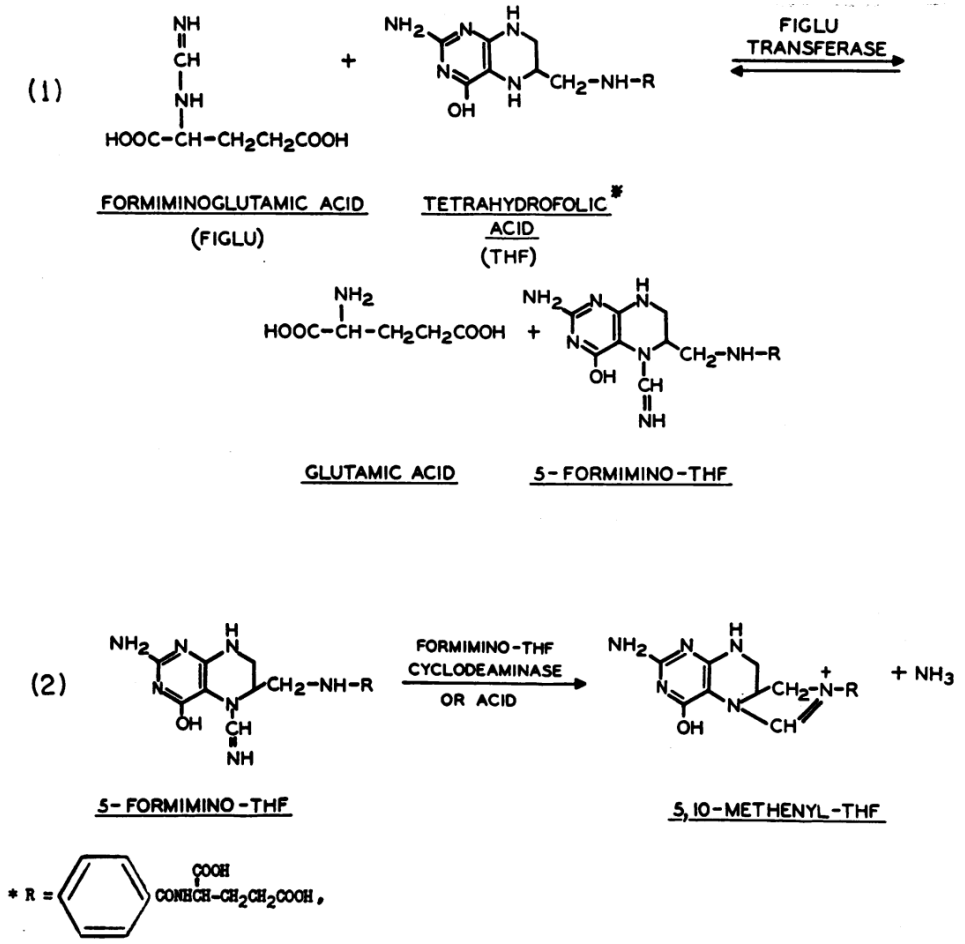


1.3.2 Catabolism of FIGLU – based on the human medical literature

After the generation of FIGLU from dietary histidine, folate-dependent reactions are required

for the removal of FIGLU from body tissues.[183] Formiminotransferase-cyclodeaminase (FTCD) is a bi-functional enzyme responsible for the next steps in the histidine catabolism pathway.[186] The transferase domain of FTCD ('FIGLU transferase' in Figure 3) catalyses the transfer of a formimino group (−CH=NH) from FIGLU to THF, to form 5-formiminotetrahydrofolate (5-formiminoTHF) and glutamate, as shown in Figure 3.[186] The cyclodeaminase domain of FTCD ('formimino-THF cyclodeaminase' in Figure 3) then catalyses a cyclodeamination of the formimino group, resulting in the production of 5,10-methenyl-THF and the release of ammonia (NH_3).[186]

Figure 3. Chemical reactions involved in the conversion of FIGLU to glutamate, 5,10-methenyl-THF, and ammonia (NH_3). From page 825 of Tabor & Wyngarden (1958).[187]



The enzymatic breakdown of FIGLU by FTCD is reported to occur exclusively in the liver.[188] Experimental studies in lab animals have demonstrated FTCD to be present in substantially

higher concentrations in liver tissue than all other tissues of the body.[189] Such findings have led to the conclusion that FTCD is a liver-specific enzyme.[189,190]

THF is required as a substrate for FTCD in order for these reactions to proceed.[185] Thus by accepting the formimino group, THF plays an important role in the degradation of FIGLU.[185] As such, in folate-deficient states, FIGLU cannot be metabolised at the normal rate and accumulates in the body.[183]

1.3.3 Catabolism of FIGLU – based on the small animal veterinary literature

The degradation pathway of FIGLU does not appear to have been specifically investigated in in cats and dogs. However, orthologs of human FTCD, the enzyme primarily responsible for FIGLU catabolism in humans, have been documented in cats and dogs.[191,192] In non-human species, the name 'formimidoyltransferase-cyclodeaminase' is preferentially used over 'formiminotransferase-cyclodeaminase' to formally classify the protein.[191,192] However the two terms are used synonymously in the scientific literature, and so hereafter both enzymes will be abbreviated to FTCD.[193]

The amino acid sequences of FTCD have not been directly compared in cats, dogs and humans. However, sequence alignment analysis of human FTCD against pig, mouse and chicken orthologs have demonstrated strong homology.[194] The amino acid sequences of these orthologous proteins were found to be 75% identical, suggesting that the FTCD proteins are highly conserved amongst eukaryotes.[194] Based on the high level of amino acid conservation amongst eukaryotes, it appears likely that the FTCD enzyme displays similar catalytic activity in cats and dogs as it does in humans.

Inferring from FTCD's activity in humans, the author predicts that in cats and dogs FIGLU is catabolised by folate-dependent reactions, catalysed by feline and canine FTCD.

1.3.4 Use of FIGLU as a functional indicator of folate deficiency

The FIGLU excretion test and other variations of FIGLU testing are based on the principle that FIGLU metabolism is a folate-dependent process.[195] Just as folate plays an important role in various one-carbon metabolic pathways, FIGLU catabolic reactions rely on folate serving as a cofactor to mediate the transfer of a one-carbon unit, the formimino residue.[195]

When folate deficiency is present, there are insufficient amounts of THF available to allow FIGLU degradation to be carried out at an adequate rate.[183] Therefore in folate-deficient states, FIGLU accumulates in the body and is excreted unaltered in large quantities in urine.[183]

FIGLU measurement therefore gives an indication of whether folate status is sufficient to enable active folate derivatives to serve their roles as cofactors in one-carbon metabolism.[196] As such, FIGLU is a metabolic indicator of folate status.[149]

1.3.5 FIGLU excretion test in humans

It has been demonstrated in humans and various animal species that folate deficiency is associated with an increased excretion of FIGLU in urine, and that the extent of urinary FIGLU excretion parallels the degree of folate deficiency.[195] A good correlation has been shown between hepatic folate concentrations and urinary excretion of FIGLU in humans.[197] In the field of human medical research, urinary FIGLU excretion is a well-described functional test for folate status that was frequently used by researchers in the 1960s and 1970s.[198]

Prior to serum folate and RBC folate assays becoming streamlined and their reference intervals refined through a plethora of scientific data, urine FIGLU tests were even used routinely in the clinical diagnostic setting.[197] A 1966 article reported: “Tests of folic-acid

function are widely used in the investigation of patients with megaloblastic anaemia. These tests are the urinary excretion of formiminoglutamic acid and the direct assay of the serum (or whole blood) concentration of various forms of folate by microbiological assay".[197] In the years that followed, significant research was carried out to refine the cut-off values for serum and RBC folate, until a point was reached where direct serum and RBC folate were considered good estimates of tissue folate status.[98,142] Once confidence was gained in the use of serum and RBC folate as estimators of folate status, this negated the need for tests like urinary FIGLU excretion and plasma homocysteine in the clinical setting.[183]

The FIGLU excretion test involved a 24-hour collection of urine that typically followed 'histidine loading'.[183] Since FIGLU is an intermediate metabolite in histidine degradation, subjects were frequently challenged with large doses of L-histidine administered orally during the test.[199] After 24 hours of urine collection, FIGLU was measured in the urine via microbiological assay, enzyme assay, high-voltage electrophoresis, conventional voltage electrophoresis, chromatographic techniques or spectrophotometric techniques.[198,200] One clinical pathology textbook reports an upper reference limit for urinary FIGLU excretion after histidine loading as being 200 μmol/24h.[183]

While once considered a valuable research tool, the requirement for 24-hour urine collection makes the FIGLU excretion test poorly suited for routine use.[11,183] FIGLU excretion tests are no longer routinely used in human medicine, likely because other functional markers (such as homocysteine) provide less labour-intensive alternatives.[149,183]

1.3.6 Reasons for the traditional use of urine FIGLU over blood FIGLU measurement

FIGLU measurement has traditionally been performed on urine, rather than blood.[183] This is because the concentration of FIGLU is typically far greater in urine, than in the blood of the same corresponding individual.[201,202] While extremely sensitive LC-MS/MS techniques now

exist that are able to detect minute amounts of FIGLU in body fluids, historically FIGLU testing relied on less advanced techniques that were unable to detect FIGLU when present in smaller quantities.[201,203] The presence of FIGLU in urine at significantly higher concentrations was therefore a key advantage exploited by researchers, given the instruments available to them.[201]

Active excretion of FIGLU in the renal tubules is the likely cause of disparity between urine and blood FIGLU concentrations.[201] Based on their investigations of human patients with marked elevations in urine FIGLU, Duran *et al*. (1981) concluded that: "tubular secretion plays a role in the excretion of this compound [FIGLU]".[201] Furthermore, experimental studies into the renal handling of organic acids have confirmed that organic acids are actively secreted into the proximal tubule.[204]

1.3.7 Specificity of the FIGLU excretion test for folate deficiency

Increased FIGLU excretion is considered specific to folate deficiency, with the exception of a few rare circumstances.[205] The inborn error of metabolism, glutamate formiminotransferase deficiency, causes a marked elevation in blood and urine FIGLU concentrations, in the absence of any deficiencies in folate.[206] This rare autosomal recessive disorder has been described in humans with an estimated incidence of around 1 in 46 000, however is yet to be recognised in any animal species.[207]

Elevations in FIGLU excretion have also been reported in rare cases of severe cobalamin deficiency and liver disease.[208,209,210] The vast majority of human patients with cobalamin deficiency do not display increased FIGLU excretion; in fact, several publications present the FIGLU excretion test as an effective diagnostic test for the differentiation of cobalamin deficiency from folate deficiency.[211,212,213] One such study by Marín *et al*. (1997) reported a

specificity of 92% for the ability of the FIGLU excretion test to discriminate between folate deficiency and cobalamin deficiency in a group of 62 patients with megaloblastic anaemia.[213]

There are sporadic reports, however, of patients with severe cobalamin deficiency demonstrating increased urinary FIGLU excretion in the presence of normal blood folate concentrations.[209] A likely explanation for this scenario is the 'methyl trap hypothesis', which suggests that severe cobalamin deficiency can cause a functional folate deficiency due to a reduction in cobalamin-dependent methyltransferase activity.[214] An impaired ability to convert 5-MTHF to other biologically active forms of folate is hypothesised to lead to a decline in folate-dependent reactions.[214]

Abnormal FIGLU excretion has also been reported in human patients with liver disease, particularly alcohol-related hepatic cirrhosis.[188,210,215] Merritt *et al*. (1962), Carter *et al*. (1961) and Knowles *et al.* (1963) excluded folate deficiency as the primary cause of abnormal FIGLU excretion results in some of their patients with liver disease, by demonstrating a persistence of abnormal FIGLU excretion results following folic acid supplementation.[188,210,215] Knowles *et al*. additionally documented normal serum folate concentrations in several of their subjects.[188]

Two major theories have been postulated for the development of abnormal FIGLU excretion in patients with liver disease.[198] Firstly, severe liver dysfunction may lead to the defective catabolism of FIGLU by the liver-specific enzyme, FTCD.[210] Since the enzymatic breakdown of FIGLU by FTCD is thought to occur exclusively in the liver, it is proposed that impaired liver function, and thus reduced FTCD activity, could directly lead to excessive FIGLU.[189,210]

Secondly, hepatic dysfunction might lead to a defect in the liver enzymes responsible for converting 5-MTHF to physiologically active forms of folate.[215] Knowles *et al*. thereby proposed that liver failure could cause a state of functional folate deficiency, within which

body folate stores are adequate, however the folate cannot be utilised due to an inability to metabolise folate into the biologically active form.[188]

The majority of patients with liver disease that have demonstrated abnormal FIGLU excretion in the literature have been those with hepatic cirrhosis.[188,210,215] In fact, Carter *et al*. exclusively reported on patients with alcohol-related hepatic cirrhosis, and presented 30 patients that fit this inclusion criteria.[215] A more varied group of patients was presented in Knowles *et al*.'s study, although 28 of 37 patients were still diagnosed with hepatic cirrhosis; 10 of these being alcohol-related hepatic cirrhosis.[188] The remaining nine patients were diagnosed with a variety of other liver diseases, such as infectious hepatitis, and drug- and toxin-induced liver injury.[188] To the author's knowledge, Knowles *et al*.'s retrospective study is the only article that describes a substantial group of patients with elevated FIGLU excretion caused by liver conditions other than hepatic cirrhosis. It therefore appears uncommon for other liver diseases to cause abnormal FIGLU excretion; Merritt *et al*. propose that this may be because liver dysfunction of a sufficient degree and for a sufficient duration may be required to impact FIGLU metabolism.[188]

1.3.8 FIGLU excretion test in the small animal veterinary literature

Urinary FIGLU excretion testing was utilised in three laboratory studies assessing folate status in cats and dogs.[124,155,196] In all three studies, the urinary FIGLU excretion test results were consistent with results obtained from other folate diagnostic tests.[124,155,196]

First, in the previously described experimental study by Thenen and Rasmussen (1978), urinary FIGLU excretion was substantially higher in cats fed a folate-deficient diet for 154 days compared to control cats fed a balanced diet.[124] At the conclusion of the experiment, a urinary FIGLU excretion test was performed after histidine loading, in addition to measurement of liver folate, plasma folate, and RBC folate, and aspiration of bone

marrow.[124] A single dose of L-histidine (0.22 g/kg) was administered subcutaneously for histidine loading and then urine collected 24 hours later.[124] FIGLU measurement was performed on the urine via an enzymatic spectrophotometric method, and a FIGLU:creatinine ratio calculated.[124,187] Urine FIGLU concentrations were markedly higher in all cats fed the folate-deficient diet (9 – 14.2 µmol/mg of creatinine), compared to control cats (undetectable - 0.0125 µmol/mg of creatinine; Table 3).[124] In two control cats, there was no FIGLU detectable at all, and in one only trace quantities were detected (0.0125 µmol/mg of creatinine).[124]

Table 3. Liver folate concentrations and urinary FIGLU excretion test results in cats fed control and folate-deficient diets in Thenen and Rasmussen's 1978 experimental study.[124]

Cat number	**Liver folate concentration (µg/g)**	**Urinary FIGLU excretion (µmol/mg of creatinine)**
Fed control diet		
72	6.05	< 0.0005
98	6.32	0.0125
100	4.39	< 0.0005
Fed folate-deficient diet		
87	0.86	9.0
99	0.54	13.4
101	0.61	14.2

Liver folate concentrations were also markedly lower in cats fed the folate-deficient diet (0.54 - 0.86 µg/g) compared to control cats (4.39 - 6.32 µg/g).[124] Similarly, plasma folate and RBC folate concentrations were considerably lower in the folate-deficient diet group compared to the control group.[124] Finally, bone marrow aspirates collected at the end of the study demonstrated marked abnormalities in cats in the folate-deficient diet group (marked megaloblastic changes to erythroid precursors), whereas bone marrow smears on the control cats were all within normal limits.[124] These results strongly suggest that cats in the folate-

deficient diet group demonstrated whole-body folate deficiency by the end of the experiment, whereas cats in the control group maintained adequate body folate levels.[124]

Urinary FIGLU excretion results in Thenen and Rasmussen's experiment thereby supported the presence of folate deficiency in one group of cats and adequate folate status in another, which appears accurate based on other testing performed in the study.[124] Urinary FIGLU excretion testing was therefore able to discriminate between folate-deficient cats and cats with adequate folate status in this experiment.[124]

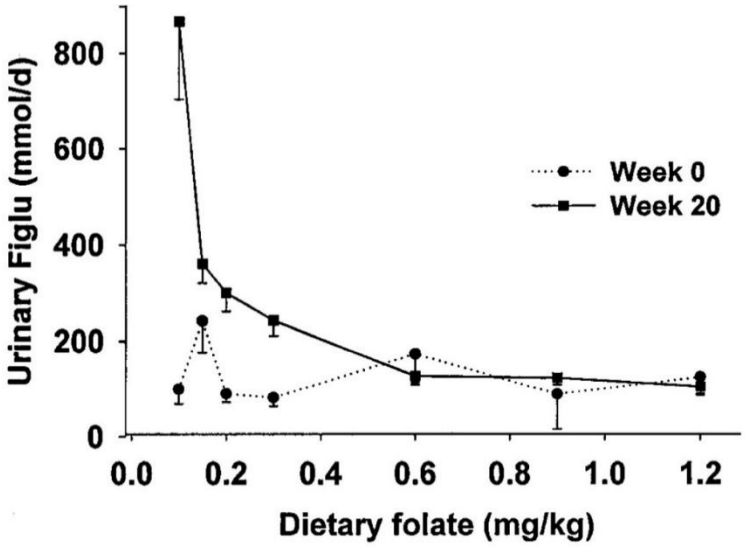
Second, a laboratory study by Yu & Morris (1998) performed urinary FIGLU excretion tests on kittens receiving purified diets with different concentrations of folic acid, as a means to assess dietary folate requirements in cats.[196] Seven experimental diets were prepared, which contained folic acid at the following concentrations: 0.1, 0.15, 0.2, 0.3, 0.6, 0.9 and 1.2 mg/kg.[196] At 10 weeks of age, 56 kittens were randomly allocated to receive one of the seven diets for 20 weeks.[196] Urinary FIGLU excretion tests were performed at zero and 20 weeks, in addition to haematology, and whole blood, plasma and RBC folate measurement.[196] For the urinary FIGLU excretion tests, urine collection was undertaken for 48 hours after the oral administration of a single dose of L-histidine at 0.22 g/kg.[196] Urine FIGLU measurement was performed via the same enzymatic spectrophotometric method.[187,196]

Dietary folic acid content had a significant effect on whole blood, plasma and RBC folate concentrations, with these values all being significantly lower at week 20 in kittens receiving experimental diets with lower folic acid content ($P < 0.01$).[196] Dietary folate also had a significant effect on urinary FIGLU excretion.[196]

Kittens receiving diets that contained folate at less than 0.6 mg/kg demonstrated significantly higher urinary FIGLU excretion ($P < 0.01$).[196] Mean urinary FIGLU excretion was over 800 mmol/24h in the group of kittens receiving 0.1 mg/kg dietary folate, whereas the mean was

consistently below 200 mmol/24h for each group receiving 0.6 mg/kg dietary folate and above (Figure 4).[196] Mean MCV was also found to be significantly higher in groups receiving below 0.3 mg/kg dietary folate ($P < 0.01$).[196] A reference interval was not provided for MCV based on the haematology analyser used in this study, however, so although a significant difference between groups was observed, it cannot be concluded whether kittens in the more folate-restricted groups displayed true macrocytosis.[196]

Figure 4. Urinary FIGLU excretion of kittens in Yu & Morris' study fed purified diets with varying folic acid content. Each point marks the mean urinary FIGLU excretion for eight kittens and the vertical bar represents the standard error of the mean. From page 2607S of Yu & Morris (1998).[196]



Yu & Morris (1998) concluded that: "The fact that urinary FIGLU excretion in folate-deficient kittens increased after a histidine load indicates that the catabolic pathway of histidine in cats is similar to that of other animals, and the load test is useful in the diagnosis of folate deficiency in kittens".[196] Furthermore, the authors considered the similar patterns observed in urinary FIGLU excretion and MCV an indication of these tests being "sensitive indices of folate status of kittens".[196]

In the absence of laboratory-specific reference intervals being reported in this study for blood folate, urine FIGLU and haematologic parameters, and without overt clinical signs, the author

cannot definitively conclude if the kittens receiving 0.1 mg/kg dietary folate were truly folate-deficient. The marked differences observed between the 0.1 mg/kg dietary folate group and the remaining groups in whole blood/plasma/RBC folate, urinary FIGLU excretion, and MCV, do however raise suspicion for the possibility of this group experiencing subclinical folate deficiency. This study thereby provides some additional evidence to suggest that urinary FIGLU excretion may be higher in folate-deficient cats, than cats with adequate folate status.[196]

A third laboratory study utilised urinary FIGLU excretion tests to screen for evidence of folate deficiency in dogs receiving phenytoin, an anti-epileptic drug implicated in possible folate deficiency-related haematologic complications in humans.[155] Twelve Beagles were randomly allocated to a treatment group and a control group; four were allocated to the control group and eight allocated to receive phenytoin for 54 weeks.[155] All dogs received a balanced diet formulated to contain folic acid at the minimum concentration recommended for adult maintenance in dogs.[155]

At 54 weeks, a urinary FIGLU excretion test was performed on all dogs.[155] Dogs received a single oral dose of L-histidine (220 mg/kg), prior to urine being collected for 24 hours.[155] Urine FIGLU measurement was performed utilising the same enzymatic method, and urine FIGLU was expressed as urine FIGLU:creatinine ratios.[155,187]

The following tests were additionally performed on all dogs at the beginning and end of the experiment: haematology, bone marrow cytology, plasma folate, RBC folate and hepatic folate measurement (via liver biopsy). Haematology, bone marrow cytology, plasma folate and RBC folate were also performed every two to three weeks in all dogs for the 54-week duration of the study.[155]

Plasma and RBC folate concentrations did significantly decrease in both the control and treatment groups ($P < 0.02$).[155] The authors postulated that this was likely the result of dietary folate concentrations being restricted to minimal concentrations.[155] However, hepatic folate concentrations demonstrated no substantial change at the end of the experiment.[155] Additionally there were no clear differences in hepatic folate concentrations between the treatment and control groups.[155] There were also no changes observed in haematology and bone marrow cytology in all dogs, and no substantial differences in urinary FIGLU excretion between groups.[155] Mean urinary FIGLU excretion in the control and treated groups were only 0.159 and 0.099 µmol/mg of creatinine respectively (Figure 5).[155]

Figure 5. Mean (± standard deviation) urinary FIGLU excretion (µmol/mg of creatinine) in Beagles receiving phenytoin for 54 weeks and control dogs. From page 1867 of Bunch *et al* (1990).[155]

Group	Value (µmol of FIGLU/mg of creatinine)
Control (n = 4)	0.159 ± 0.13
Treated (n = 8)	0.099 ± 0.09

As hepatic folate measurement is regarded as the gold-standard test for assessing folate deficiency, and dogs in both groups did not display any substantial reduction in hepatic folate concentrations during the experiment, one can infer that the treated dogs did not appear to be suffering from a whole-body folate deficiency at the end of this study.[155] One can therefore surmise that in this study, dogs that were not deficient in folate displayed low urinary FIGLU excretion.[155] This study therefore provides some evidence to suggest that the urinary FIGLU excretion test may be able to recognise dogs with adequate whole-body folate status.[155]

Urinary FIGLU excretion results for cats and dogs in the aforementioned studies that were deemed to have adequate folate status, were also similar to results reported for healthy humans.[124,155,216] Normal urinary FIGLU excretion in adult humans after an L-histidine load (198 mg/kg) has been reported as 0.005 – 1.08 µmol/mg creatinine.[216] Thus the urinary

FIGLU excretion results measured in these studies for cats and dogs deemed to have adequate folate status, fits within the reference interval cited for humans (range in cats: undetectable – 0.0125 µmol/mg creatinine; means in dog groups: 0.099 and 0.159 µmol/mg creatinine).[124,155,216]

In summary, two laboratory studies demonstrated that kittens with suspected experimentally induced folate deficiency displayed significantly higher urinary FIGLU excretion than kittens with adequate folate status.[124,196] A third laboratory study found that adult dogs that did not appear to have a whole-body folate deficiency, excreted very small amounts of urine FIGLU.[155] To date, urinary FIGLU excretion tests have not been performed to evaluate naturally occurring folate deficiency in cats and dogs.

1.3.9 Spot measurement of FIGLU in human urine and plasma

There are clear practical advantages to the performance of spot measurement of FIGLU rather than FIGLU excretion tests that require 24-hour urine collection. While FIGLU measurement is no longer routinely performed to assess for folate deficiency, it is an important component of the diagnostic investigation for a particular inborn error of folate metabolism, glutamate formiminotransferase deficiency (FTCD deficiency).[206] FTCD deficiency, also termed 'formiminoglutamic aciduria' (FIGLU-uria), is an autosomal recessive disorder wherein deficiency of the FTCD enzyme impairs normal FIGLU metabolism, resulting in elevated FIGLU concentrations in the urine, blood and tissues.[207] Historically, the syndrome of FIGLU-uria was diagnosed via 24-hour urinary FIGLU excretion tests.[216] However more recently, spot measurements of FIGLU in urine and plasma have replaced the use of 24-hour urinary FIGLU excretion tests in the diagnostic confirmation of FIGLU-uria.[206]

In a 2019 study, spot plasma and urine FIGLU measurements were reported in 18 children with FIGLU-uria.[206] Plasma FIGLU was measured within an acylcarnitine profile analysis using tandem mass spectrometry (MS/MS).[206] A normal plasma FIGLU was considered 0 μmol/L, and results in affected children ranged from 0.47 to 7.69 μmol/L.[206] Urine FIGLU results were also reported in mg/g creatinine, and a normal result considered to be undetectable quantities. Urine FIGLU measurements in the children with FIGLU-uria ranged from 16.6 to 259.4 mg/g creatinine.[206]

This data indicates that with the use of highly sensitive MS/MS equipment, in certain situations the spot measurement of FIGLU in the plasma and urine of subjects with defective FIGLU catabolism may be a suitable substitute for 24-hour urinary FIGLU excretion tests. To the author's knowledge, the use of FIGLU spot measurements have not been evaluated for the investigation of folate deficiency.

CHAPTER 2: DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR QUANTITATIVE MEASUREMENT OF FORMIMINOGLUTAMIC ACID IN CANINE AND FELINE PLASMA

2.1 INTRODUCTION

Alterations to folate status are widely recognised in cats and dogs with gastrointestinal disease.[129,172,173] Low blood folate concentration (hypofolataemia) is reported in up to 32% of dogs and up to 40% of cats with chronic enteropathies.[77,78] The clinical significance of hypofolataemia in small animals, however, remains unclear.

In human medicine, the laboratory finding of hypofolataemia is considered consistent with a diagnosis of folate deficiency, which is treated with folic acid supplementation.[98] Folate deficiency is where negative folate balance has reached the stage of reduced folate tissue stores in the body.[139] A good correlation has been demonstrated between folate concentrations in the liver (the primary site for folate storage in the body), and serum folate concentrations in human patients.[140,141] Significant efforts have been directed to developing cut-off values of serum folate at which whole-body folate deficiency is most likely to be present in human patients.[142]

In humans, folate deficiency is also well known to have potential haematologic, gastrointestinal and neurologic complications.[4] Studies of cats and dogs with experimentally induced folate deficiency have consistently demonstrated haematologic complications, in addition to variably demonstrating other problems like weight loss and glossitis.[124,125,126] To date, only a single publication exists that documents haematologic complications from naturally occurring folate deficiency in a small animal, and this was a case report describing two cats fed an unconventional folate-deficient vegan diet.[16] These studies suggest that a sufficiently severe state of folate deficiency can result in clinical changes in cats and

dogs.[16,124,125,126] However, it is uncertain whether it is feasible for pet cats and dogs fed a nutritionally balanced diet to inadvertently develop a whole-body folate deficiency that is severe enough to cause these clinical complications.

In veterinary medicine, there is inadequate published data to determine whether hypofolataemia is detrimental to feline and canine patients, and whether folic acid supplementation is warranted. The first step to answering these questions is to determine what serum folate concentration is associated with whole-body folate deficiency. Folate concentration of hepatic tissue collected by liver biopsy is considered the gold standard test for defining folate deficiency.[140] However, given the invasiveness of liver biopsy collection, a variety of blood tests are used as surrogate markers for liver folate stores.[140] In fact, in 2005 the WHO published revised serum folate cut-off values for determining folate deficiency in humans, which were primarily based on a study that used plasma homocysteine concentrations as a metabolic indicator for folate deficiency.[142] The cut-off value for folate deficiency was designated as the serum folate concentration below which plasma homocysteine concentrations started to increase above the normal range.[142] A 2020 study evaluating the relationship between serum homocysteine and serum folate concentrations in dogs, however, found no association between the two.[163] Thus plasma homocysteine is unlikely to be useful as a functional indicator of folate status in dogs, and to date no other metabolic markers for folate deficiency have been identified in cats and dogs.

The current author sought to explore the potential utility of plasma FIGLU as a functional indicator of folate deficiency in cats and dogs. Urine FIGLU has long been used in the research field of human medicine.[198] However, the original test described, a 24-hour urinary excretion test, was so laborious and time-consuming that it has since lost favour.[183] For the same reasons, the urinary FIGLU excretion test is not considered a practical research tool for pet cats and dogs. There have been huge advancements in analytical chemistry techniques

over the past few decades though, and analytical methods like LC-MS/MS offer such a high sensitivity for the detection of metabolites, that 24-hour urine collection may no longer be necessary.

The author proposes that spot measurement of plasma FIGLU shows promise as a functional marker of folate deficiency in cats and dogs. Further, the author hypothesised that the use of a substrate product ratio, plasma FIGLU:glutamic acid, would improve the method's ability to predict folate status. The primary objectives of the current study were to develop and analytically validate an LC-MS/MS method for the quantitative measurement of FIGLU and glutamic acid in feline and canine blood samples.

2.2 Materials and methods

2.2.1 Chemicals and reagents

L-Formiminoglutamic acid (FIGLU) standard was procured from Biosynth (CAS No. 816-90-0, Staad, Switzerland). L-Glutamic acid (Glu) standard was obtained from Sigma-Aldrich (CAS No. 56-86-0, St. Louis, United States). D5-Glutamic acid (D5-Glu) standard was sourced from Cambridge Isotope Laboratories, Inc. (CAS No. 2784-50-1, Tewksbury, United States). Bovine Serum Albumin (BSA) was obtained from Sigma-Aldrich (CAS No. 9048-46-8, St. Louis, United States), and phosphate buffered saline (PBS) 20x concentrate purchased from Rowe Scientific (Sydney, Australia). Ultra-pure water was collected from a Milli-Q Reference Water Purification System, for the preparation of PBS. HPLC-grade methanol was procured from Supelco (CAS No. 67-56-1, Bellefonte, United States) for use in the generation of standard solutions and for methanol precipitation.

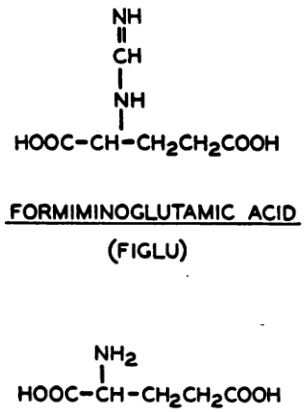
For use in the LC system as solvents, LC-MS-grade methanol and formic acid were acquired from Fisher Chemical (CAS No. 67-56-1, Pittsburgh, United States), and Ajax Finechem

(CAS No. 64-18-6, Sydney, Australia) respectively. LC-MS grade water was procured from a Sartorius Arium Water Purification System (Göttingen, Germany) for the generation of solvents.

2.2.2 Preparation of standard solutions, calibration standards and quality controls

D5-Glu was selected to serve as the internal standard (IS) for both Glu and FIGLU. While a stable isotopically labelled form of FIGLU would have been the IS of choice for FIGLU, this was not economically feasible for this project because not only is stable isotope labelled FIGLU extremely expensive, but it is also difficult to procure as it needs to be custom-made. Structural analogues are recognised as an acceptable type of IS in LC-MS bioanalysis.[217] FIGLU and Glu have similar chemical structures and physiochemical properties (including a similar level of polarity), which are important in the selection of a suitable IS (Figure 6).[217] Thus a stable isotope labelled form of Glu was deemed suitable and selected to act as the IS for FIGLU in this project.

Figure 6. Chemical structure of FIGLU and Glu. From page 825 of Tabor & Wyngarden (1958).[187]



Stock solutions were prepared of the two analytes (FIGLU and Glu), and the IS (D5-Glu) in H_2O, at 1 mg/mL. All stock solutions were stored at -80°C. Working solutions of FIGLU (2 - 25 µg/mL), Glu (300 - 800 µg/mL) and D5-Glu (2 – 20 µg/mL) were prepared by diluting stock solutions in methanol.

A 4% BSA in PBS solution was generated to serve as a surrogate matrix for the preparation of calibration standards. As both FIGLU and Glu are endogenously present in the plasma of healthy cats and dogs, analyte-free preparations of the authentic matrix are not available. The use of a surrogate matrix for calibration standards is a well described approach to the LC/MS quantitation of endogenous compounds in biological samples.[218] According to recent publications, the surrogate matrix approach continues to be a common strategy in LC-MS techniques in situations where analyte-free preparations of the authentic matrix do not exist.[219]

4% BSA in PBS was selected to serve as the surrogate matrix due to similarities in pH, ionic strength and protein concentrations as canine and feline plasma.[218] 2.5 mL of PBS 20x concentrate was diluted into 47.5 mL of ultrapure water, to generate PBS. Two grams of BSA was weighed on an analytical scale, and dissolved into 50 mL of PBS. The resulting solution of 4% BSA in PBS was aliquoted and stored at -80°C.

For the establishment of calibration curves, six levels of calibration standards were selected. The two highest calibration standards were prepared by spiking 4% BSA with working solutions of FIGLU and Glu. Four additional calibration standards were then generated by serial dilution with 4% BSA. The set of six calibration standards were spiked with a working solution of D5-Glu, to achieve an IS concentration of 200 ng/mL throughout all calibration standards. Calibration standard levels covered the concentration range of 31.25 - 1000 ng/mL for FIGLU, and 1000 – 100 000 ng/mL for Glu. Specific concentrations for each calibration

standard level are detailed in Table 4. Calibration standards were prepared in bulk, and divided into single-use aliquots of 200 µL which were stored at -80°C until use.

Table 4. Nominal concentrations of FIGLU and Glu in 4% BSA calibration standards.

Calibration standard	FIGLU concentration (ng/mL)	Glu concentration (ng/mL)
CAL 1	31.25	1000
CAL 2	62.5	2000
CAL 3	125	4000
CAL 4	250	8000
CAL 5	500	16 000
CAL 6	1000	100 000

For the analysis of healthy canine plasma in an added dilution integrity experiment, three additional calibration standards were later generated in order to account for the lower concentrations of plasma FIGLU observed in healthy canine plasma. These calibration standards were produced by the serial dilution of CAL 1 with a 4% BSA solution that contained 200 ng/mL D5-Glu, to maintain the IS concentration at 200 ng/mL. These three calibration standards contained FIGLU at concentrations of 15.625 ng/mL, 7.8125 ng/mL, and 3.90625 ng/mL; and Glu at concentrations of 500 ng/mL, 250 ng/mL, and 125 ng/mL.

Blank samples were prepared in bulk by spiking 4% BSA with 10 µg/mL D5-Glu, to achieve an IS concentration of 200 ng/mL in the 4% BSA. Neat 4% BSA was used to act as double blank samples. Blank and double blank samples were divided into 200 µL aliquots, which were stored at -80°C.

Feline and canine quality controls (QCs) were established using pools of feline and canine plasma respectively, sourced from leftover patient samples submitted to Veterinary Pathology Diagnostic Services (VPDS) of The University of Sydney. Given that sample submissions to VPDS are largely comprised of patients visiting referral services at the University Veterinary

Teaching Hospital Sydney (UVTHS), these pools can be considered to include predominantly sick patients, ranging in severity from outpatients with mild illnesses to critically ill patients hospitalised in the intensive care unit. As the ultimate goal is to eventually test predominantly sick hypofolataemic animals, it was deemed appropriate to validate the method using plasma from ill animals.

QCs were prepared at two concentration levels in feline plasma and canine plasma respectively. Feline and canine QCs were generated by dividing feline and canine plasma pools each into two fractions, then spiking one of the two pools with FIGLU and Glu, to produce a feline high QC (HQC), feline low QC (LQC), canine HQC and canine LQC pool. All QCs were additionally spiked with the IS, D5-Glu, to obtain a concentration of 200 ng/mL. Each QC pool was divided into 200 µL aliquots and stored at -80°C until the time of analysis.

A pool of plasma was also generated from a group of clinically normal dogs, which was used to perform additional dilution integrity experiments. The pool of healthy canine plasma was spiked with FIGLU and D5-Glu to generate a canine healthy QC pool. The decision was made to add dilution integrity experiments on healthy canine plasma after preliminary results indicated that plasma from ill cats and dogs performed poorly in dilution integrity experiments, and this was suspected to be due to matrix effects that were amplified in ill patients.

2.2.3 Sample preparation

Plasma and surrogate matrix samples were thermally equilibrated to room temperature. In an Axygen 1.5 mL plastic snaplock microcentrifuge tube (Union City, United States), 800 µL of methanol was mixed with 200 µL of plasma or surrogate matrix to initiate protein precipitation. The sample was vortexed for 15 seconds, then allowed to sit at room

temperature for 5 minutes to homogenise. Samples were centrifuged in an Eppendorf Centrifuge 5415 D (Hamburg, Germany) at 13 200 rpm for 5 minutes. The resulting supernatant was transferred to a Thermo Scientific glass screw top autosampler microvial (Waltham, United States), in preparation for injection into the LC system for analysis. This protocol of sample preparation was based on an LC-MS/MS method published by Piraud *et al.* (2011), which demonstrated the qualitative measurement of FIGLU within a broader project that simultaneously analysed 67 molecules of biological interest for the diagnosis of inborn errors of metabolism.[220]

During method development, urine, serum and plasma samples were tested to guide selection of the sample matrix of choice. As anticipated, urine FIGLU concentrations were dramatically higher than serum and plasma FIGLU concentrations. However, FIGLU was endogenously present at detectable concentrations in serum and plasma using the LC-MS/MS method. As urine FIGLU concentrations could additionally be affected by extraneous factors that alter the renal handling of FIGLU, plasma and serum were considered superior matrices for the evaluation of folate status. Given that the LC-MS/MS method was adequately sensitive to measure plasma and serum FIGLU, these were therefore selected over urine FIGLU. Plasma was ultimately selected as the ideal matrix, because it offers the advantage over serum of avoiding coagulation-induced alterations in analyte concentrations.[144] When clotting occurs during the preparation of serum, the coagulation process can change the concentration of certain blood constituents.[144] As coagulation does not occur during the preparation of plasma, this eliminates the possibility of analyte concentrations having been altered by the coagulation process.[144] Heparinised plasma was therefore selected as the preferred sample matrix for the LC-MS/MS method.

2.2.4 LC-MS/MS instrumentation

LC-MS/MS was performed using a Shimadzu HPLC system (Kyoto, Japan) coupled to an AB Sciex QTRAP 5500 triple quadrupole mass spectrometer (Framingham, United States). The HPLC system consisted of a Shimadzu LC-30AD pump and a Shimadzu SIL-30AC autosampler. Chromatographic separation was performed using an Agilent Polaris NH2 180 Å HPLC column (Santa Clara, United States), with a particle size of 3 µm, column length of 50 mm and column diameter of 2.0 mm. The column was maintained at 40°C within a Shimadzu CBM-20A column oven. Sciex OS software (Version 3.1.6.44, AB Sciex) was used for data acquisition, processing, and analysis.

2.2.5 MS method development and optimisation

Fragmentation analysis and optimisation of MS parameters were performed using the infusion-based guided optimisation feature in Sciex OS software. Guided multiple reaction monitoring (MRM) was performed in the "MRM Infusion" mode, and optimised parameters were used to generate a new MRM acquisition method for FIGLU, Glu and D5-Glu.

Stock solutions of FIGLU, Glu and D5-Glu were infused directly into the mass spectrometer via a syringe pump, for the guided optimisation of each compound. After Q1 scans confirmed the identification of each compound's parent ion, the optimisation tool was set to find the three most intense product ions generated during MRM of each compound. Optimisations were performed in both positive and negative ionisation modes, to determine which polarity generated the most intense peaks. Positive ionisation mode was ultimately determined to produce the best results and therefore selected for the final MS method.

For FIGLU and D5-Glu, the MS method was tuned with the selection of product ions with the greatest intensities, in order to maximise sensitivity in the measurement of FIGLU, which is known to exist at very low concentrations in biological fluids. For Glu the MS method was

detuned, with the selection of a product ion that produces a lower intensity. Glu exists in plasma at significantly higher concentrations than FIGLU. The detuning of Glu reduces the difference in peak areas between Glu and FIGLU, assisting in the generation of a single MS method for the quantitation of both compounds. In addition to a product ion being selected for the quantitative measurement of each compound, the 'quantifier ion', a second product ion was selected to act as a qualifier ion for each compound.

Declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimised for each product ion, by the automated MRM optimisation tool. The optimised product ions and optimised MS parameters (DP, CE and CXP) for FIGLU, Glu and D5-Glu were used to generate a new MS method.

2.2.6 Mass spectrometric conditions

Samples were ionised using an electrospray ionisation (ESI) source, in positive ionisation mode. The ESI source was operated at a temperature of 450°C, curtain gas of 25 psi, ion source gas 1 of 40 psi, ion source gas 2 of 40 psi, and spray voltage of 5500V. Data was acquired in MRM mode and mass transitions of precursor/product ions for quantitative measurement of the compounds of interest were set to: 175.127/84.054, 148.065/102.056, and 153.094/88.078 for FIGLU, Glu and D5-Glu respectively. Optimised MS parameters for each mass transition are detailed in Table 5.

Table 5. MS parameter settings. Details of the optimised parameters for each mass transition, in the analysis of FIGLU, Glu and D5-Glu.

Compound	Q1 mass	Q3 mass	Dwell time (ms)	DP (V)	Entrance potential (V)	CE (V)	CXP (V)
FIGLU Quantifier	175.127	84.054	100	65	10	28	10
FIGLU Qualifier	175.127	56.067	100	65	10	44	7
Glu Quantifier	148.065	102.056	100	65	10	15	12
Glu Qualifier	148.065	56.074	100	65	10	40	15
D5-Glu Quantifier	153.094	88.078	100	60	10	23	39
D5-Glu Qualifier	153.094	107.058	100	60	10	15	50

2.2.7 Chromatographic conditions

An LC method was developed and optimised to achieve good separation of the three compounds, which demonstrate relatively high polarity.[221,222] Normal phase and hydrophilic interaction liquid chromatography (HILIC) techniques of separation were tested using the Agilent Polaris NH2 column, which contains a polar stationary phase. Stock solutions of FIGLU, Glu and D5-Glu were injected onto the column, while sequentially testing different mobile phases at varying gradient elutions.

Ultimately a normal phase chromatographic method was selected, with the mobile phases comprised of: 0.1% formic acid in water (A); and methanol (B). The gradient elution program was set as follows: 95% mobile phase B for the first minute; B was then reduced linearly to 10% over 4 minutes; B was held at 10% for one minute; B was then escalated linearly to 95% in 0.1 minutes; and B was then held at 95% for 1.9 minutes. An injection volume of 5 µL was employed. HPLC separation was carried out at a constant flow rate of 0.4 mL/min throughout analysis, and the total analytical run time was 8 minutes.

2.2.8 Sample order in analytical series and data processing

A blank and double blank sample, calibration standards, and QC samples were analysed at the beginning and end of each analytical run. Calibration standards and QC samples were always injected in a sequence of increasing concentrations. Two solvent blanks containing 100% methanol were injected after every two samples.

Raw LC-MS/MS data files were processed using Sciex OS software, and automated peak detection and peak integration performed using the MQ4 algorithm. Peak integration was performed with the processing method adjusted to the following settings: Gaussian smooth width of 7.0 points, peak splitting factor of two points, and noise percentage of 40%. The expected retention times were set to 3.654 minutes for FIGLU, 4.053 minutes for Glu and 3.990 minutes for D5-Glu. Following data processing, peaks were additionally visually inspected to confirm that all peaks had been adequately integrated. The resulting quantitative data was inspected for potential outliers, and outliers were excluded from the final analysis.

2.2.9 Method validation

Analytical validation was performed on the developed method with respect to linearity, accuracy, precision, recovery and dilution integrity.

2.2.9.1 Calibration curve and linearity

Analyte:IS response ratios were used for the calculation of calibration standard concentrations. Specifically, the chromatographic peak area of FIGLU or Glu were divided by the peak area of D5-Glu, to determine concentrations for the calibration curve. Six point calibration curves were constructed by plotting the analyte:IS response ratio against the nominal concentration of the corresponding calibration standard, and employing a linear regression with appropriate weighting.

Linearity was assessed for FIGLU and Glu by analysing three replicates of each level of calibration standard, and evaluating the resulting correlation coefficient (r^2). For the remaining method validation experiments, calibration curves were generated on each day of analysis, based on a single measurement of each calibration standard. Only calibration standards with accuracy within 15% of the nominal concentration were included in daily calibration curves.

2.2.9.2 Within-run and between-run accuracy and precision

The within-run accuracy and precision of the method for the measurement of FIGLU and Glu were evaluated by analysing six replicates at four QC levels in feline and canine plasma, within a single analytical run. Accuracy was calculated as [accuracy (Δ%) = ((mean of measured concentrations) – expected concentration) * 100]. Percent coefficient of variation (% CV) was used to express precision. Accuracy was regarded as the degree of closeness between the measured value and the nominal value; whereas precision was viewed as how close individual measurements were to each other.[223] Between-run accuracy and precision of FIGLU and Glu were determined by measuring four QCs in three independent runs over three consecutive days. Six replicates of each QC were analysed each day.

Within-run and between-run accuracy were required to be within 15% of the expected concentration at each QC level, and within-run and between-run precision was required to not exceed 15%, as recommended by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation.[223,224]

2.2.9.3 Recovery

Recovery was assessed to confirm that the extraction process selected for this method was reproducible and efficient.[223] Recovery of FIGLU was determined by comparing measurements obtained from a canine plasma sample spiked with FIGLU and D5-Glu before

extraction (pre-extraction spiked samples), against a canine plasma sample spiked after extraction (post-extraction spiked samples). Recovery was determined by: the calculated concentration of FIGLU in the pre-extraction spiked sample, expressed as a percentage of the calculated concentration of FIGLU in the post-extraction spiked sample.

Recovery of FIGLU was also evaluated on surrogate matrix samples, in order to assess for parallelism between the surrogate matrix and canine plasma. Recovery was assessed by comparing results from pre-extraction spiked surrogate matrix samples, against post-extraction spiked surrogate matrix samples. All samples were analysed in duplicate.

2.2.9.4 Dilution integrity

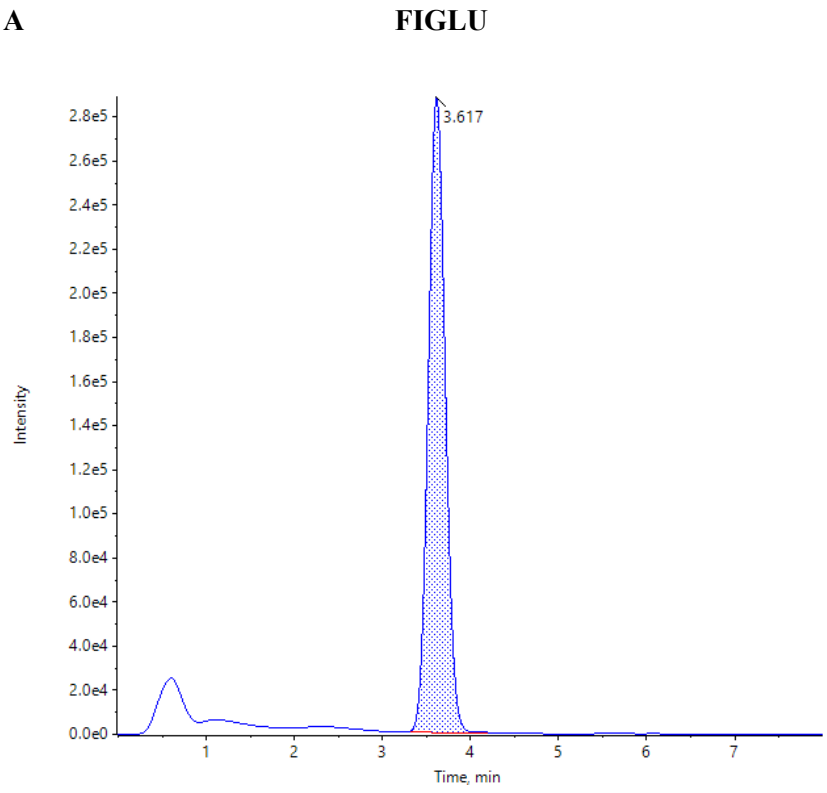
Dilution integrity for FIGLU and Glu were evaluated by the sequential dilution of: a spiked ill canine plasma sample (canine HQC), a spiked ill feline plasma sample (feline HQC), and a spiked healthy canine plasma sample (healthy canine QC), with the surrogate matrix (4% BSA). First, surrogate matrix was spiked with D5-Glu to achieve an IS concentration of 200 ng/mL. Second, 200 µL of the IS-spiked surrogate matrix was combined with 200 µL of canine HQC, in order to obtain a 1:2 dilution. The procedure was repeated to achieve a 1:4 dilution of canine HQC, and a 1:8 dilution. The same procedure was repeated for the feline HQC and the healthy canine QC. Three replicates of each dilution were analysed in a single run. Surrogate matrix was used for the serial dilution rather than canine or feline plasma, because of the endogenous presence of FIGLU and Glu in any authentic plasma sample and the requirement for an analyte-free matrix to achieve a true serial dilution of FIGLU and Glu. FDA and EMA guidelines for bioanalytical method validation recommend that the mean accuracy of the diluted samples should be within 15% of the expected concentration.[223,224]

2.3 RESULTS

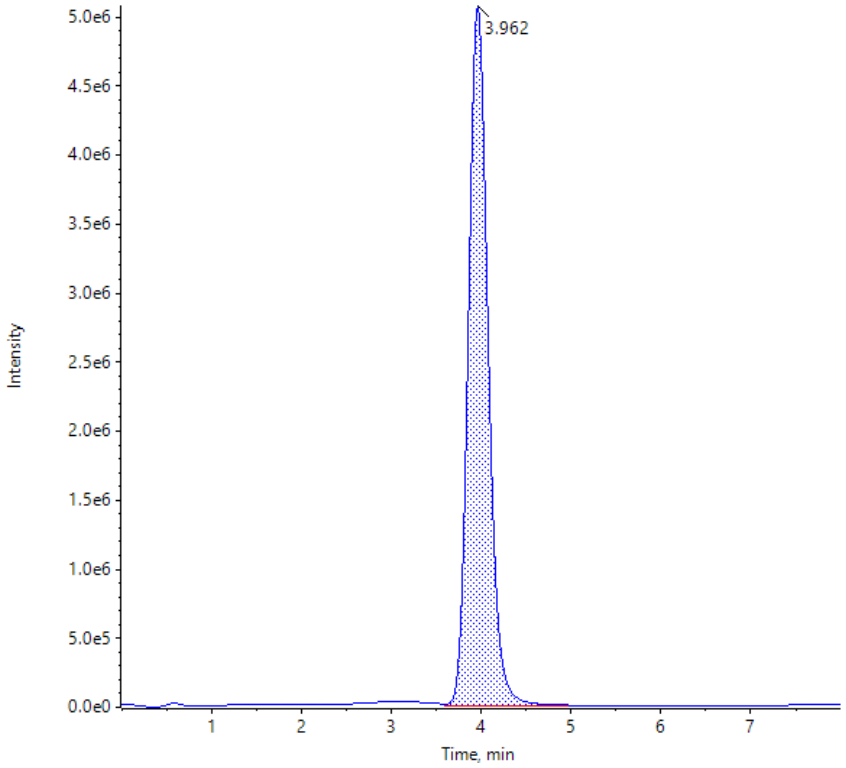
2.3.1 Method development

Using the optimised method, FIGLU and Glu eluted with well resolved peaks of adequate peak shape in feline and canine plasma, as illustrated in Figure 7. The method was able to detect small amounts of FIGLU that were endogenously present in pooled feline and canine plasma samples. The endogenous FIGLU concentrations of the ill feline and canine plasma pools were determined to be 75 ng/mL and 490 ng/mL respectively. The endogenous Glu concentrations of the pooled ill feline and canine plasma samples were determined to be 102 500 ng/mL and 93 500 ng/mL respectively.

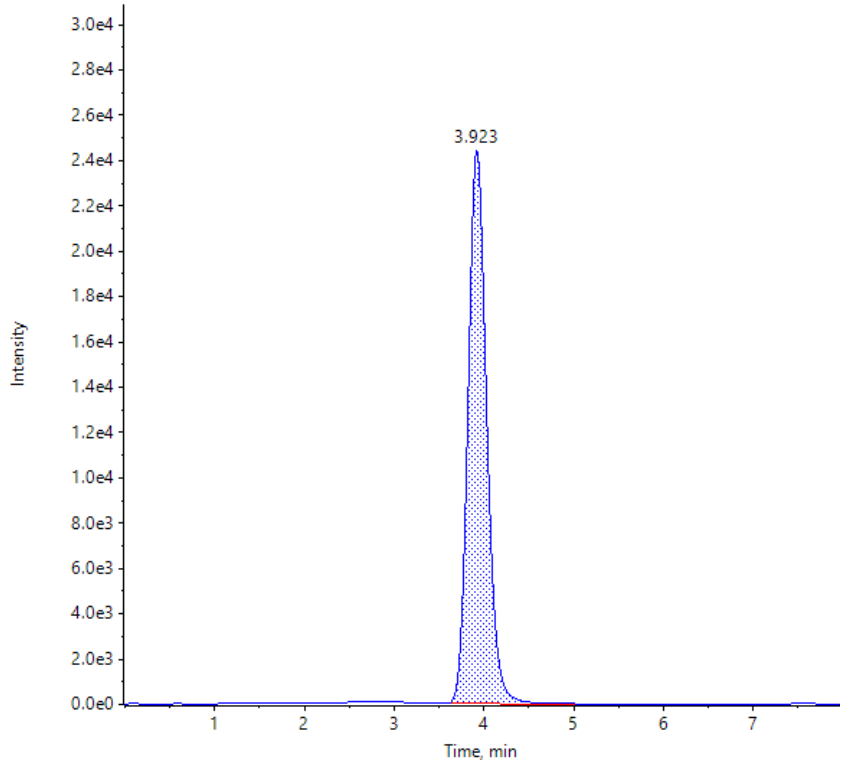
Figure 7. Representative chromatograms of FIGLU, Glu and D5-Glu obtained by the reported LC-MS/MS method. Extracted ion chromatograms are displayed of: (A) Endogenous FIGLU peak in canine plasma with a retention time of 3.60 minutes, (B) Endogenous Glu peak in canine plasma with a retention time of 3.95 minutes, and (C) D5-Glu peak (as the IS) in canine plasma with a retention time of 3.90 minutes. Total ion chromatograms of endogenous FIGLU, endogenous Glu and spiked D5-Glu in (D) Canine plasma, and (E) Feline plasma, are also presented.



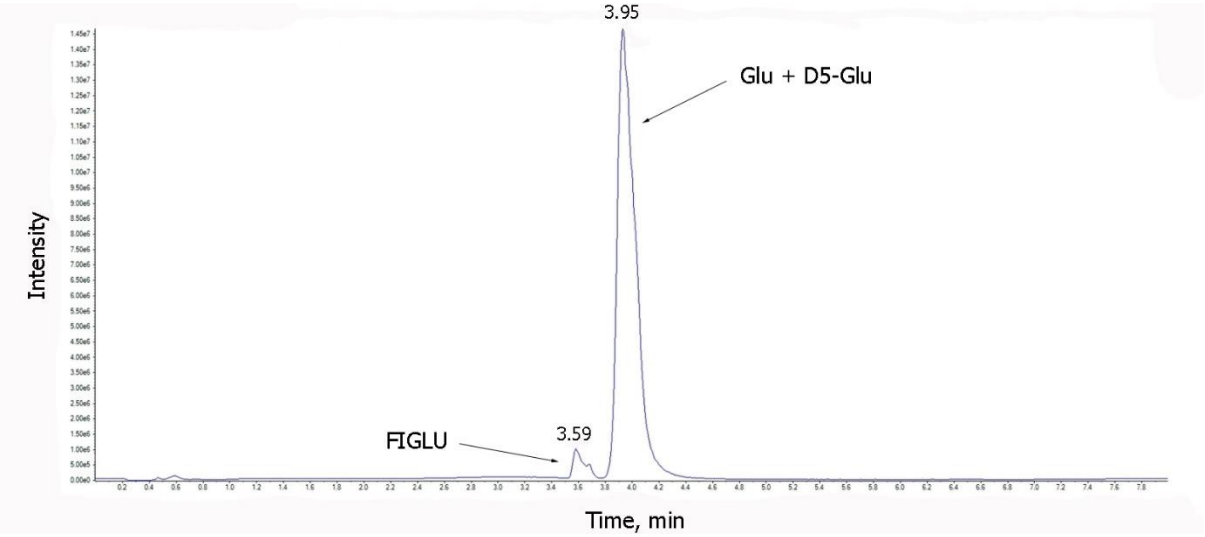
B **Glutamic acid**



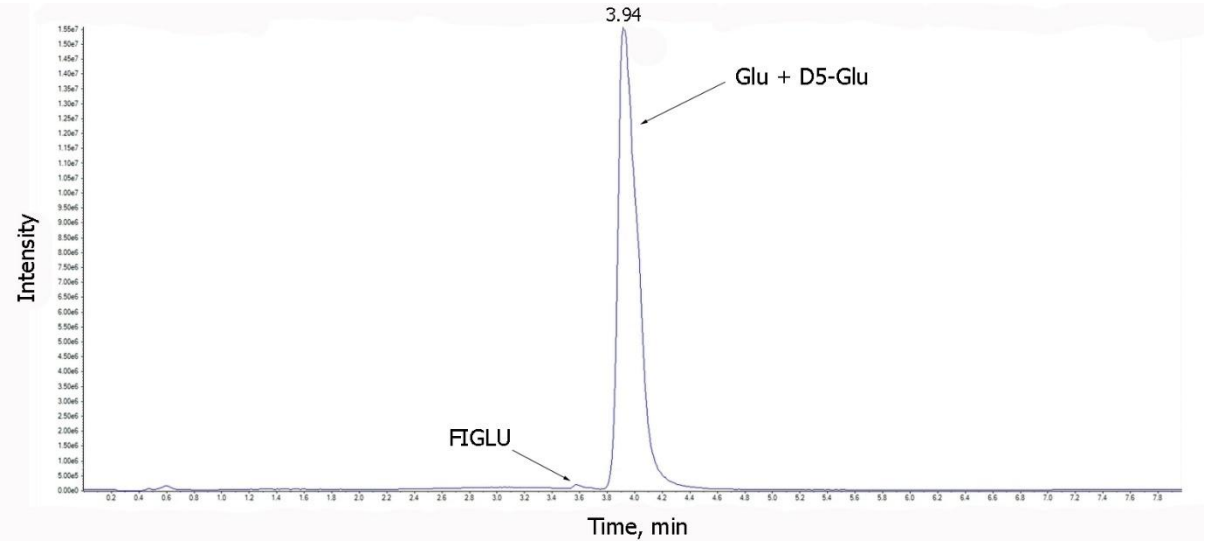
C **D5-Glu**



D



E

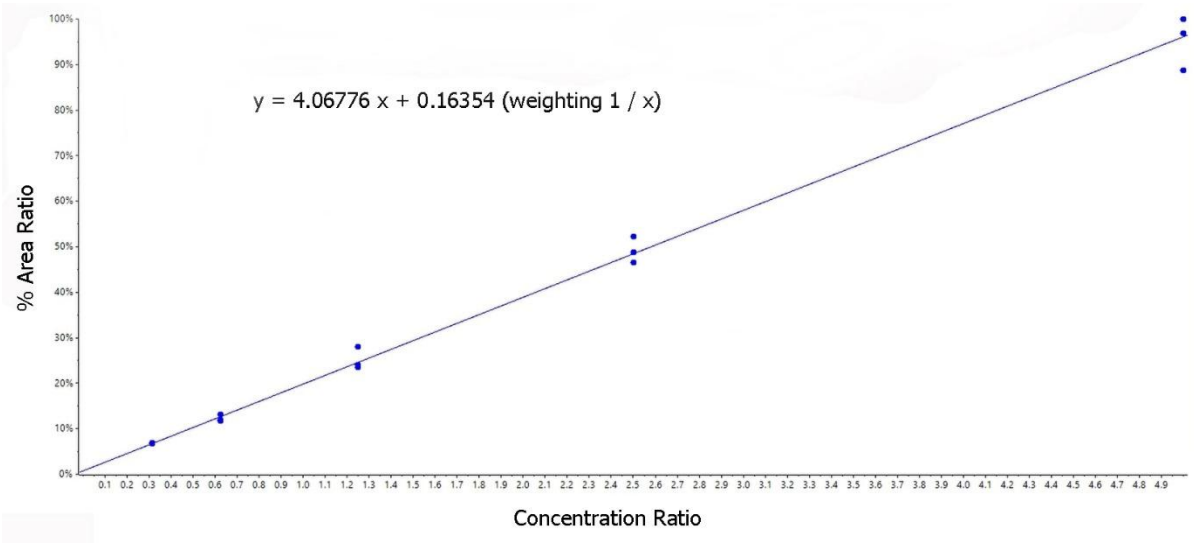


2.3.2 Linearity

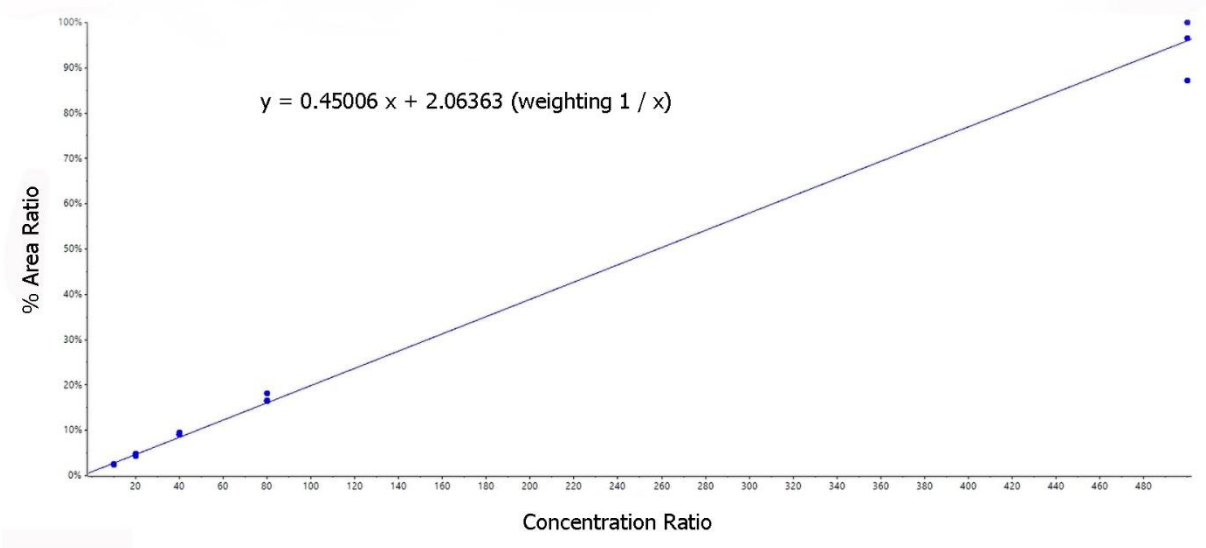
Linearity was established for the calibration curves of FIGLU and Glu over the concentration ranges of 31.25 to 1000 ng/mL, and 1000 ng/mL to 100 000 ng/mL respectively. A weighting factor of 1/x was selected to achieve the curve of best fit. Using this weighted linear model, a correlation coefficient (r^2) of 0.994 was observed for both FIGLU and Glu. Calibration curves generated from the linearity study are illustrated in Figure 8.

Figure 8. Calibration curves of FIGLU (A) and Glu (B) obtained during the linearity experiment.

A



B



Based on the physiologic range of FIGLU reported in human plasma (36.5 - 273.4 ng/mL) and the FIGLU concentrations observed in ill feline and canine pooled plasma samples (75 and 490 ng/mL), this calibration range (31.25 -1000 ng/mL) was expected to be adequately wide so as to cover most healthy feline and canine samples.[203] It was only once healthy feline and canine plasma samples were collected and tested (Chapter 3), that it was discovered that

healthy small animal plasma often demonstrates FIGLU concentrations far below 31.25 ng/mL. For this reason, additional calibration standards were then generated with lower FIGLU concentrations, for use on healthy small animal plasma samples.

A calibration range of 1000 to 16 000 ng/mL had originally been chosen for Glu based on previously reported physiologic ranges in canine and feline plasma. A range of 2075 to 7680 ng/mL has been documented for plasma Glu in a group of clinically normal dogs.[225] Feline plasma Glu has been shown to range from 3578 ng/mL up to 6180 ng/mL in one group of clinically normal cats, and up to 23 541 ng/mL in a group of clinically normal cats in a second study.[226,227] However, preliminary data from this current project demonstrated Glu concentrations of 90 000 to 105 000 ng/mL in ill feline and canine plasma pools; thus the upper end of the calibration range was extended from 16 000 ng/mL to 100 000 ng/mL.

2.3.3 Accuracy and precision

The within-run and between-run accuracy and precision data for the measurement of FIGLU and Glu are presented in Tables 6 and 7. All within-run and between-run accuracy and precision values for measurement of FIGLU and Glu in feline and canine plasma met the predefined validation requirements.

The within-run accuracy for FIGLU measurement ranged between 1.89% and 9.02% from the nominal concentrations, and within-run precision for FIGLU ranged between 2.06% and 6.83%. Between-run accuracy for FIGLU measurement ranged from 1.83% to 11.07% from the nominal value, and between-run precision for FIGLU ranged between 4.19% and 10.86%.

Within-run accuracy for the measurement of Glu ranged from 0.26% to 1.68%, and within-run precision values for Glu were between 1.68% and 3.76%. The between-run accuracy for Glu ranged from 0.24% to 2.44% from the expected concentration, and between-run precision for Glu was between 5.49% and 6.05%.

Based on these results it can be concluded that the method is accurate and precise for the quantitative measurement of FIGLU and Glu in feline and canine plasma.

Table 6. Within-run and between-run precision and accuracy for FIGLU in feline and canine plasma.

QC level	Nominal concentration (ng/mL)	Mean ± SD	Accuracy (%)	Precision (% CV)
Within-run variation for FIGLU				
Feline LQC	75	73.58 ± 2.84	-1.89	3.86
Feline HQC	175	159.22 ± 3.28	-9.02	2.06
Canine LQC	490	506.95 ± 34.62	3.46	6.83
Canine HQC	740	792.00 ± 22.83	7.03	2.88
Between-run variation for FIGLU				
Feline LQC	75	76.38 ± 5.42	1.83	7.10
Feline HQC	175	162.40 ± 6.80	-7.20	4.19
Canine LQC	490	519.32 ± 54.02	5.98	10.4
Canine HQC	740	658.08 ± 71.48	-11.07	10.86

Table 7. Within-run and between-run precision and accuracy for Glu in feline and canine plasma.

QC level	Nominal concentration (ng/mL)	Mean ± SD	Accuracy (%)	Precision (% CV)
Within-run variation for Glu				
Feline LQC	102 500	103 502.10 ± 2448.87	0.98	2.37
Feline HQC	105 500	105 778.46 ± 1777.74	0.26	1.68
Canine LQC	92 000	93 547.18 ± 3515.01	1.68	3.76
Canine HQC	98 000	99 514.63 ± 1768.12	1.55	1.78

Between-run variation for Glu				
Feline LQC	102 500	102 257.46 ± 6127.10	-0.24	5.99
Feline HQC	105 500	102 930.93 ± 5845.79	-2.44	5.68
Canine LQC	92000	92327.48 ± 5065.12	0.36	5.49
Canine HQC	98000	98418.21 ± 5958.67	0.43	6.05

2.3.4 Recovery

The recovery of FIGLU in canine plasma was 92.5%, and recovery of FIGLU in the surrogate matrix (4% BSA) was 92.2%. The selected sample preparation method therefore demonstrates excellent extraction efficiency and is well suited to the quantitative measurement of FIGLU. The similar recovery results obtained for authentic matrix (plasma) and surrogate matrix (4% BSA) also suggest that extraction efficiency is similar between the two matrices.

2.3.5 Dilution integrity

Results from the dilution integrity experiments are presented in Tables 8 and 9. Dilution integrity was confirmed for Glu up to a 1:8 dilution, with accuracy being within 12% of the nominal value at all concentrations. Dilution integrity was not demonstrated for FIGLU, particularly in plasma samples from ill cats and dogs. A feline HQC sample diluted 1:8 displayed an accuracy 81.11% from the nominal concentration, whereas the acceptance criteria is 15%. A canine HQC sample diluted 1:8 similarly demonstrated an accuracy 61.82% from the expected concentration. The healthy canine QC sample performed much better with dilution, displaying an accuracy of FIGLU 27.5% from the nominal concentration at a 1:8 dilution. While FIGLU accuracy in the healthy canine QC still did not meet the FDA and EMA-recommended acceptance criteria for dilution integrity, the results were far closer to these goals than in ill patient samples.[223,224]

Precision of FIGLU and Glu measurement in the dilution QC samples did meet the acceptance criteria, ranging from 0.18 to 6.29%, which was similar to the precision observed in the original QC samples (0.12 to 5.72%). Diluting the plasma QC samples with surrogate matrix therefore did not appear to affect precision of the method. This is suggestive of parallelism being present between plasma and the surrogate matrix, supporting the use of 4% BSA in the calibration standards.

Table 8. Results of the dilution integrity experiment for the measurement of FIGLU.

Sample	Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Accuracy (%)	Precision (% CV)
Ill canine plasma				
Canine HQC	740	741.27		5.72
1:2 dilution canine HQC	370	405.15	9.50	1.05
1:4 dilution canine HQC	185	239.90	29.67	0.51
1:8 dilution canine HQC	92.5	149.69	61.82	2.85
Ill feline plasma				
Feline HQC	330	331.85		1.06
1:2 dilution feline HQC	165	194.69	17.99	3.48
1:4 dilution feline HQC	82.5	115.43	39.91	0.35
1:8 dilution feline HQC	41.25	74.71	81.11	3.21
Healthy canine plasma				
Healthy canine QC	100	100.28		1.05
1:2 dilution healthy canine QC	50	56.48	12.97	1.77
1:4 dilution healthy canine QC	25	31.04	24.15	0.20
1:8 dilution healthy canine QC	12.5	15.94	27.50	3.94

Table 9. Results of the dilution integrity experiment for the measurement of Glu.

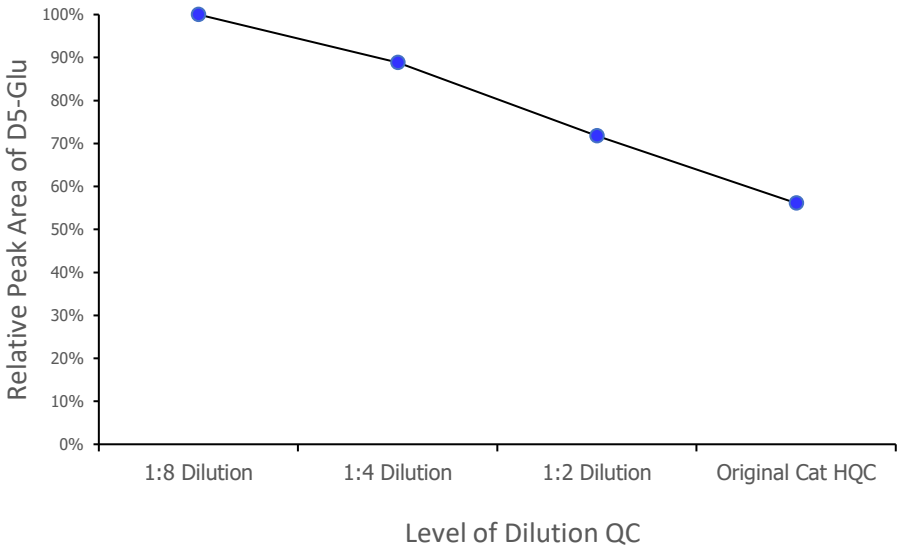
Sample	Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Accuracy (%)	Precision (% CV)
Ill canine plasma				
Canine HQC	113 000	113 068.25		2.96
1:2 dilution canine HQC	56 500	57 096.07	1.05	0.49
1:4 Dilution canine HQC	28 250	28 776.94	1.87	1.74
1:8 dilution canine HQC	14 125	14 061.02	-0.45	1.83
Ill feline plasma				
Feline HQC	130 000	130 469.01		1.46
1:2 dilution feline HQC	65 000	68 330.17	5.12	0.33
1:4 dilution feline HQC	32 500	34 209.75	5.26	0.64
1:8 dilution feline HQC	16 250	17 232.65	6.05	1.26
Healthy canine plasma				
Healthy canine QC	3830	3829.19		0.12
1:2 dilution healthy canine QC	1915	1893.11	-1.14	0.41
1:4 dilution healthy canine QC	957.5	889.68	-7.08	0.18
1:8 dilution healthy canine QC	478.75	421.94	-11.87	6.29

2.3.6 Ion suppression of the internal standard and analytes

The cause of accuracy problems in the dilution QCs became clear upon inspection of the IS peak areas observed across the range of dilutions. In spite of the IS being added at the same concentration across all samples, the peak area of D5-Glu measured lower in the less diluted samples, which had a more concentrated matrix with a higher concentration of Glu and other

unidentified compounds (Figure 9). This pattern is strongly suggestive of ion suppression of the IS.

Figure 9. Peak area of D5-Glu measured in various dilution QCs comprising feline plasma, all containing D5-Glu at the same concentration (200 ng/mL).

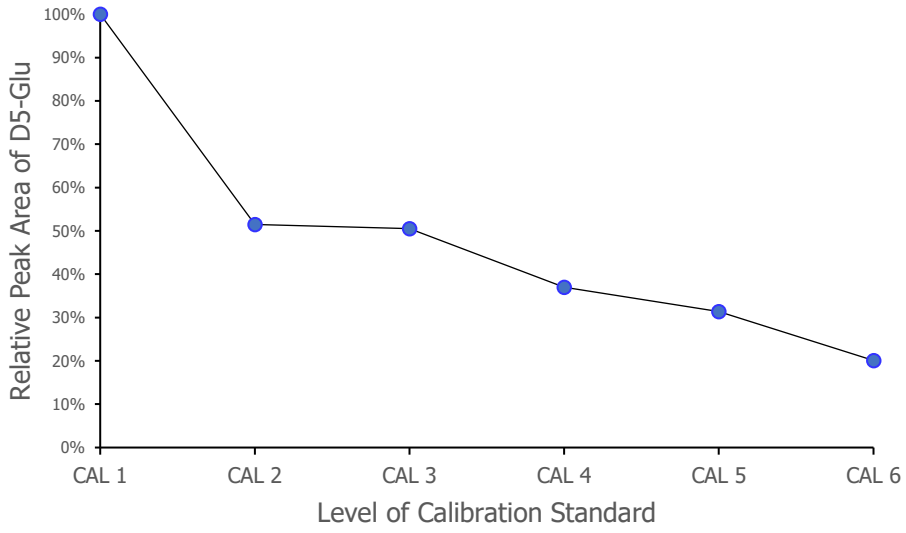


A similar pattern was observed in the calibration standards, whereby the peak area of D5-Glu progressively decreased as the standards became more concentrated (Figure 10). As the calibration standards comprised a simpler surrogate matrix of 4% BSA in PBS, with the only added compounds being Glu, FIGLU and D5-Glu, and because Glu was spiked at the highest concentrations of the three, the most likely source of ion suppression in the calibration standards was determined to be Glu.

In the dilution integrity experiments, the peak areas of both Glu and D5-Glu appeared to be affected by ion suppression to a similar extent, resulting in all dilution QCs demonstrating an accurate calculated concentration for Glu. The calculated concentrations for FIGLU, however, were significantly higher than the expected concentrations in the dilution QCs. The peak areas of FIGLU also showed a smaller decline than expected with each dilution, and this disparity to the nominal concentrations was more dramatic than the changes observed in the

peak areas of D5-Glu upon dilution. It was hypothesised that FIGLU experienced ion suppression to a greater extent than D5-Glu and Glu, accounting for the contrasting accuracy results between FIGLU and Glu in the dilution integrity experiments.

Figure 10. Peak area of D5-Glu measured in the six calibration standards, all containing D5-Glu at the same concentration (200 ng/mL).



When healthy canine plasma samples were diluted, FIGLU results were more accurate than when ill patient samples were diluted. The pools of ill patient plasma also demonstrated dramatically higher Glu concentrations than the healthy canine plasma. It was concluded that the massively increased Glu concentration in the plasma of ill patients likely enhanced ion suppression. The differing FIGLU accuracy results between healthy and ill animal plasma therefore support the theory that ion suppression caused by high concentrations of Glu is the major cause of poor dilution integrity performance.

Ion suppression is a common problem in LC-MS/MS analysis.[228] In some cases, when ion suppression affects an analyte and its stable isotope labelled IS to the same degree, incorporation of the IS can mitigate any detrimental effects of ion suppression on accuracy of the method. Ion suppression could have a greater impact on accuracy, however, when an IS is

used that is not identical to the analyte; such is the case with our use of D5-Glu as an IS for FIGLU in this method. Use of an IS that is not chemically and structurally identical to the analyte, could result in disparate levels of ion suppression affecting each compound.

During method optimisation it was discovered that increasing the frequency of methanol blanks reduced the detrimental impact of ion suppression on the precision and accuracy of FIGLU measurement. When methanol blanks were run less frequently, it was discovered that the peak area of FIGLU would significantly fall for replicates analysed more than two samples after the last solvent blank. The peak area of D5-Glu did not demonstrate the same drop, however, resulting in imprecise and inaccurate calculated concentrations for FIGLU during these latter scans. It was hypothesised that Glu molecules and other compounds likely become adhered to the lines during the analysis of samples, and these increase the number of co-eluting compounds competing with FIGLU for ionisation. When methanol blanks are run, this rinses the lines of adhered compounds, so that there is a smaller number of compounds competing with FIGLU for ionisation. The increased frequency of methanol blanks was integrated into the final LC-MS/MS method, with two methanol blanks run after every two scans of plasma or surrogate matrix samples. While this technique reduced the frequency of markedly erroneous results caused by ion suppression, it did not resolve the problems observed with ion suppression compromising accuracy in the dilution integrity experiments.

2.4 Discussion

In the study reported herein, an LC-MS/MS method for the quantitative measurement of plasma FIGLU in cats and dogs was developed and analytically validated. Precision and accuracy of the described method for the measurement of FIGLU in undiluted plasma samples met validation requirements recommended by the FDA and EMA guidelines for

bioanalytical method validation.[223,224] The reported method was therefore found to be precise and accurate for the quantitative measurement of FIGLU in undiluted plasma.

At the outset of the project, it was unknown whether spot plasma FIGLU concentrations would be high enough in healthy animals, to be quantifiable via LC-MS/MS. Prior to recent advancements in analytical techniques, particularly before the evolution of hyphenated mass spectrometry, FIGLU was undetectable in the blood of healthy human patients.[202] The first time a reference interval for blood FIGLU was published in the human medical literature was in 2006: the reported range being 37 to 273 ng/mL in plasma.[203]

The current study detected small amounts of FIGLU in canine and feline plasma, using the described method. Endogenous FIGLU concentrations in pools of plasma from ill cats and dogs were found to be 75 ng/mL and 490 ng/mL respectively. These results fall close to the cited reference interval for FIGLU in human plasma.[203]

Preliminary data from this study suggest that 4% BSA in PBS is an appropriate surrogate matrix for canine and feline plasma, for the generation of calibration curves for the measurement of FIGLU. The surrogate matrix and plasma samples appeared to behave in similar ways with respect to extraction recovery, precision and ion suppression. For quantitative LC-MS/MS analysis, calibration standards are ideally prepared using the same matrix as the study samples being quantified.[223,224] In situations where the compound of interest is present endogenously in the study matrix and analyte-free preparations of the authentic matrix do not exist, however, use of a surrogate matrix for calibration standards is a well-described approach to circumvent this issue.[218] While preliminary results from this current study support the use of 4% BSA in PBS as a surrogate matrix, a more extensive set of parallelism experiments could be considered for future studies, to provide additional objective scientific justification for the use of 4% BSA in PBS in calibration standards.

Ion suppression was a key issue raised during the current study's validation experiments. The IS, D5-Glu, experienced significant ion suppression, which appeared to be at least partly caused by another compound of interest in the study, Glu. During LC-MS/MS analysis, all compounds in the sample matrix compete for ionisation by the ion source, including those compounds that will never actually be measured.[228] While the author suspects that ion suppression in this study was primarily caused by Glu, there may have also been contributions from other unidentified and unmeasured compounds co-eluting with the target compounds.

The current investigator was able to reduce the negative impact of ion suppression on the precision and accuracy of plasma FIGLU measurement by making adjustments to the method's batch setup design. However, these adjustments to the method were unable to prevent accuracy problems from being observed during FIGLU measurement in dilution experiments. The calculated concentrations of FIGLU in the dilution QCs were significantly higher than their expected concentrations. This contrasted to results obtained for Glu in the dilution integrity experiments, which maintained a high level of accuracy throughout all dilution QCs.

The author postulates that this occurred because Glu and D5-Glu experienced ion suppression to a similar extent, highlighting the way that use of an analyte's stable isotope labelled IS can effectively compensate for matrix effects and other variations incurred on an analyte during LC-MS/MS analysis. In contrast, FIGLU appeared to experience differing levels of ion suppression compared to the IS, leading to very poor accuracy in FIGLU measurement during the dilution experiments.

The author hypothesises that using a stable isotope labelled analogue of FIGLU may eliminate these accuracy problems. However, unfortunately the cost of stable isotope labelled

FIGLU is so high, that it was not an economically viable option for the current study. For future studies, accuracy could be optimised and a more robust method produced if stable isotope labelled FIGLU were used.

One of the objectives of the study was to evaluate whether the use of a substrate product ratio, plasma FIGLU:Glu, would improve the method's ability to estimate folate status and thereby provide a more robust metabolic marker of folate deficiency. Data obtained from the current study, however, suggest that a plasma FIGLU:Glu ratio generated using the described method is unlikely to yield improved results over plasma FIGLU measurement alone. Of key importance, the current study found that FIGLU and Glu experienced differing levels of ion suppression, which appeared to affect the final calculated concentrations of each compound. Assuming that FIGLU and Glu measurements obtained from a single plasma sample have been potentially affected by ion suppression to different extents, comparing FIGLU against Glu in a ratio would be inappropriate.

Further, Glu measurements obtained on the ill feline and canine plasma pools suggest that it is not uncommon for plasma Glu concentrations to be markedly elevated in ill cats and dogs. Plasma Glu measurements obtained on ill feline and canine plasma pools using this method were over 90 000 ng/mL, which is four times greater than the upper reference limit reported for cats, and over ten times greater than the upper end of the range reported in groups of healthy dogs.[225,227,229] Studies have documented higher plasma Glu concentrations in dogs with hepatocellular carcinoma and IBD, however elevations were mild, with the highest recorded plasma Glu result in these studies being 17 000 ng/mL.[225,229]

It should be noted that most mass spectrometers are not designed to quantify higher concentrations like this, and the linear dynamic range of analytes using this particular MS analyser is typically cited as being below 1000 ng/mL (equivalent to 5000 ng/mL when 1:5

dilutions are used in sample preparation).[230] Thus, while the author predicts that these ill patient samples do truly have profoundly elevated plasma Glu concentrations that are in the tens of thousands of ng/mL, the exact Glu results obtained in these samples should not be expected to be as accurate as measurements far lower, within the machine's dynamic range. Additionally, the specific calibration standard concentrations of Glu selected in this research project were not ideally suited for the ill patient samples that unexpectedly displayed markedly elevated Glu concentrations. This was a limitation of the current study, and validation experimental results obtained for Glu may have been impacted by the Glu concentrations used in the calibration standards. As it had already become clear that comparing FIGLU against Glu would not be helpful and the use of FIGLU:Glu ratios would not be pursued for the next portion of the research project, it was decided that reformulating the calibration standards to better suit the high Glu concentrations of ill patients was not necessary.

Elevated plasma Glu concentrations should not result from folate deficiency, nor any other factors directly related to folate status. The only change in plasma Glu concentrations that one might theoretically see with folate deficiency is decreased plasma Glu due to reduced conversion of FIGLU to Glu by impaired folate-dependent reactions. Given that marked changes in plasma Glu concentrations unrelated to folate status appear common in ill cats and dogs, this could negate the potential benefits of use of the substrate product ratio plasma FIGLU:Glu in the assessment of folate status. The author recommends that further research focuses on the use of plasma FIGLU as a functional marker of folate deficiency, rather than use of plasma FIGLU:Glu ratios.

2.5 CONCLUSIONS

In conclusion, the current author developed and analytically validated an LC-MS/MS method for the quantitative measurement of plasma FIGLU and glutamic acid. The method demonstrated good precision, accuracy, linearity, and extraction efficiency. Endogenous FIGLU was successfully detected and quantified in canine and feline plasma samples, and results fell close to the cited reference interval reported for human plasma FIGLU. Spot plasma FIGLU measurement via LC-MS/MS is therefore a viable tool for the estimation of FIGLU concentrations in cats and dogs. Further investigations are warranted to evaluate the relationship between serum folate and plasma FIGLU concentrations, and to determine whether elevated spot plasma FIGLU concentrations are observed with folate deficiency and severe hypofolataemia in cats and dogs.

CHAPTER 3: FORMIMINOGLUTAMIC ACID AND FOLATE MEASUREMENT IN CLINICALLY NORMAL CATS AND DOGS

3.1 INTRODUCTION

Blood FIGLU measurement has not previously been investigated as a potential research tool in veterinary medicine. Urinary FIGLU excretion tests have, however, been utilised in laboratory studies of cats and dogs, and results from those tests were consistent with findings in historical studies that demonstrated a correlation between urine FIGLU and whole-body folate status in humans and various animal species.[124,155,195,196,197]

Qualitative and quantitative measurement of blood FIGLU are becoming increasingly common in the screening of newborn humans for inborn errors of metabolism.[206] To date, however, there are no studies reporting the measurement of blood FIGLU in a group of cats and dogs. The main objectives of this study were: 1) To report ranges of plasma FIGLU in groups of clinically normal cats and dogs; and 2) To assess for a correlation between serum folate and plasma FIGLU concentrations in clinically normal cats and dogs.

3.2 MATERIALS AND METHODS

3.2.1 Study population

Clinically normal cats and dogs between 6 months and 10 years were considered for inclusion. Subjects were staff-owned and client-owned pets at Kirrawee Veterinary Hospital in Sydney, Australia. For inclusion, subjects were required to be clinically healthy based on owner-reported history and physical examination. Animals were excluded if there was any evidence of gastrointestinal disease or other medical conditions on history or physical examination. This study was approved by The University of Sydney Animal Ethics Committee and written consent was obtained from each owner (Project number 2023/2269).

Leftover plasma from a single ill patient was additionally analysed, based on an expectation that the FIGLU concentration would be elevated in this animal. This 6 year old male neutered Maltese X Poodle dog received trimethoprim-sulfadiazine (TMS, 16 mg/kg every 12 hours) (Tribrissen, Jurox Animal Health, Rutherford, Australia) for 15 days prior to collection of this particular blood sample, as treatment for bacterial cholangitis. TMS had been initiated after the patient demonstrated a poor clinical response to both amoxicillin and enrofloxacin, and culture & sensitivity profiles on repeated cholecystocentesis samples displayed the development of resistance to amoxicillin-clavulanate and other commonly used antimicrobials, but ongoing susceptibility to TMS. Based on historical studies demonstrating that long-term administration of TMS can cause a total and/or functional folate deficiency, it was suspected that this patient might display an elevated plasma FIGLU concentration.[231] This patient did not display any adverse effects from TMS administration, and the bacterial cholangitis appeared to resolve following TMS therapy.

A serum sample was unfortunately not available for folate measurement in this patient, from the period when they were receiving TMS. International guidelines in human medicine, however, have recommended that serum or plasma samples can be used interchangeably for folate testing "without changes of result".[144] A plasma sample was therefore used in this one patient for folate measurement. To the author's knowledge, the folate assay utilised in this project has not been validated for use with plasma; therefore accuracy on this sample type cannot be verified. These limitations were taken into account when interpreting the significance of this plasma folate result.

3.2.2 Sample collection and storage

Blood was collected via jugular venipuncture using a 23-gauge, ¾-inch needle (ZebraVet, Brisbane, Australia) and a 3 mL or 5 mL syringe (Global Veterinary Products, Brisbane,

Australia). Owners were requested to fast the animal overnight prior to blood collection. Blood collection took place between February and March 2025.

Blood was collected into a lithium heparin tube and serum clot activator tube (1.3 mL screw cap micro sample tubes; Sarstedt, Nümbrecht, Germany). Blood samples in serum tubes were allowed to clot for 30 minutes at room temperature. Blood samples in both serum tubes and lithium heparin tubes were centrifuged in a StatSpin VT Centrifuge (Beckman Coulter, Brea, United States) at 15 800 rpm for 10 minutes. Plasma supernatant was transferred into an Axygen 1.5 mL plastic snaplock microcentrifuge tube (Union City, United States). Serum was transferred into a Biologix 2 mL screw cap cryotube (Shandong, China), which was wrapped in aluminium foil to protect folate from degradation caused by light exposure.

Plasma and serum samples were stored at -80°C until the time of analysis. Plasma samples from clinically normal cats and dogs were stored between one and five weeks prior to analysis, and serum samples stored for three to seven weeks before analysis. On the day of analysis, serum samples were allowed to thaw at room temperature. Immediately after thawing, all serum samples were transported directly to the commercial laboratory (Vetnostics Pathology, Sydney, Australia) on ice. Serum folate assays were initiated upon arrival of the samples. Plasma samples were thawed at room temperature immediately prior to sample preparation and analysis, which were both performed on site at The University of Sydney.

3.2.3 Analytical methods

Plasma FIGLU and Glu concentrations were measured using the LC-MS/MS method described and analytically validated in chapter 2. Samples were prepared using methanol precipitation as described in section 2.2.3. Calibration curves were generated using seven levels of calibration standards, which covered the concentration range of 3.90625 – 250

ng/mL for FIGLU, and 125 - 8000 ng/mL for Glu. Four replicates of each patient plasma sample were analysed consecutively, and all patient samples analysed in a single batch. Outlier calculated concentrations of FIGLU and Glu were excluded from further analysis. The mean calculated concentration of the four replicates was determined to be the final plasma FIGLU and Glu concentration for each patient, to be used for further statistical analysis.

Serum folate measurement was performed at a commercial veterinary laboratory (Vetnostics Pathology), by use of a solid-phase competitive chemiluminescence immunoassay (Atellica IM Analyzer, Siemens Healthineers, Erlangen, Germany). All patient samples were analysed once, in a single batch.

The Atellica IM Folate assay is a fully automated method that utilises pre-formulated reagents generated by the analyser's manufacturer to perform a series of treatment, incubation and washing steps.[232] First, 100 µL of serum is pre-treated with dithiothreitol and sodium hydroxide to release folate from endogenous binding proteins within the patient sample.[232] Second, biotin-labelled folate binding protein (FBP) and a solid phase reagent are dispensed, and avidin that is coupled to paramagnetic particles in the solid phase reagent binds to the FBP.[232] FBP thereby becomes immobilised on the solid phase. Third, acridinium-ester-labelled folate is added, which competes with folate in the patient sample for binding with the biotin-labelled FBP.[232] Fourth, the sample is washed, which eliminates compounds that have not bound to FBP on the solid phase. Finally, acid and base reagents are added to induce the chemiluminescent reaction.[232] The concentration of endogenous folate in the patient sample determines the amount of acridinium-ester-labelled folate that binds to the limited amount of FBP due to competition for binding sites, and this inverse relationship is reflected in results reported by the system.[232]

The Atellica IM Folate assay has not been validated for use in canine and feline patient samples. However, method comparison studies have been performed by the commercial laboratory, with another competitive immunoassay that has previously been analytically validated in feline and canine sera, the Immulite 2000 Folic Acid assay (Siemens Healthineers, Erlangen, Germany) (Doug Hayward BVSc FRCPath, email communication, April 2025).[151] According to Doug Hayward of Vetnostics, twenty canine serum samples and twenty feline serum samples were analysed with the Atellica IM Folate assay (Vetnostics Pathology, Sydney, Australia) and the Immulite 2000 Folic Acid assay (Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, United States) (Email communication, April 2025). Serum samples were transported from Australia to the United States under refrigeration. The coefficient of determination (R^2) was 0.87 for canine samples ($y = 2.76\ x - 21.14$), and 0.90 for feline samples ($y = 0.961\ x + 0.9942$), suggesting good correlation in serum folate measurements. The possibility of international transport negatively affecting correlation results was also considered. Bland-Altman plots were additionally evaluated and no significant bias was identified.

The Siemens Atellica IM Folate assay and Siemens Immulite Folic Acid assay utilise similar methodologies, and demonstrate similar reportable ranges (Atellica: 0.79 – 54.36 nmol/L; Immulite: 2.3 – 52 nmol/L) and similar spiking recoveries (Atellica: 87 – 116%; Immulite: 96 – 106%).[151,233] The Siemens Atellica IM Folate assay is reported by the manufacturer as having extremely high precision in human serum samples, being designed to maintain within-laboratory precision below 11% CV.[151]

A reference interval for canine and feline serum folate specific to this instrument has not yet been generated by the local laboratory and has not been reported in the veterinary literature. Given that acceptable correlation has been observed with an external reference laboratory, serum folate measurements obtained in the current study were compared against an expected

reference interval derived from the same external reference laboratory. The reference intervals utilised for this current study were 17.5 – 55.5 nmol/L (7.7 – 24.4 μg/L) for canine serum folate, and 22.0 – 49.1 nmol/L (9.7 – 21.6 μg/L) for feline serum folate.[152]

3.2.4 Statistical analysis

Statistical analyses were completed using jamovi software, version 2.6.[234] Data were assessed for normality by the examination of histograms and Q-Q plots, and performance of the Shapiro-Wilk test. Correlations between plasma FIGLU and serum folate concentrations in the clinically normal cat and dog groups were assessed by calculation of Spearman correlation coefficients. Statistical significance was set to a P value < 0.05 for all analyses.

In accordance with guidelines developed by the American Society of Veterinary Clinical Pathology (ASVCP), reference intervals were not calculated for plasma FIGLU given the small sample sizes below 20.[235] Instead reference values are reported herein, including the median, minimum and maximum values.

3.3 RESULTS

3.3.1 Study population

Eleven clinically normal dogs and 10 clinically normal cats were enrolled in the study. Ages ranged from 1 to 10 years in the dog group (median: 5 years), and 6 months to 9.5 years in the cat group (median: 6.5 years). Body weights ranged from 4.7 to 61.0 kg in dogs (median: 21.6 kg), and from 3.3 to 7.0 kg in cats (median: 4.9 kg). There were 9 male dogs (7 neutered, 2 entire) and 2 female dogs (1 spayed, 1 entire). There were 5 male neutered cats and 5 female spayed cats. The canine study population included the following breeds: mixed breed (n = 2), Staffordshire Bull Terrier (n = 2), Bernese Mountain Dog (n = 1), Chihuahua (n = 1), English Springer Spaniel (n = 1), French Bulldog (n = 1), Golden Retriever (n = 1), Kelpie (n

= 1), and Miniature Poodle (n = 1). In the feline study population, the breed distribution was as follows: Domestic Shorthair (n = 7), Oriental Shorthair (n = 1), Persian (n = 1), and Tonkinese (n = 1).

3.3.2 Plasma FIGLU concentrations in clinically normal cats and dogs

Plasma FIGLU concentrations in clinically normal dogs and cats were not normally distributed (Figures 11 and 12, respectively). Plasma FIGLU concentrations ranged from 4.5 to 21.0 ng/mL in clinically normal dogs, and from 9.1 to 57.0 ng/mL in clinically normal cats (Table 10). The median plasma FIGLU concentration was 8.20 ng/mL in dogs, and 26.24 ng/mL in cats.

Figure 11. Distribution of plasma FIGLU concentrations (ng/mL) in 11 clinically normal dogs

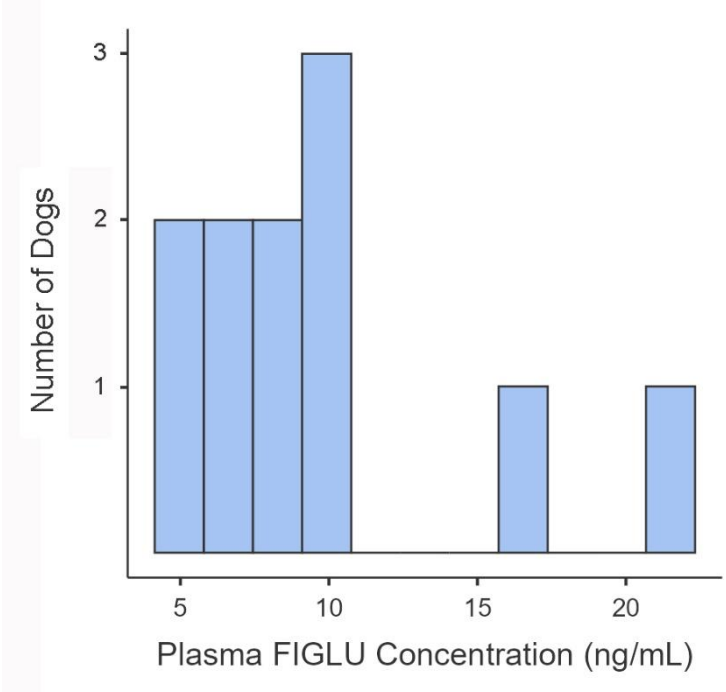


Figure 12. Distribution of plasma FIGLU concentrations (ng/mL) in 10 clinically normal cats

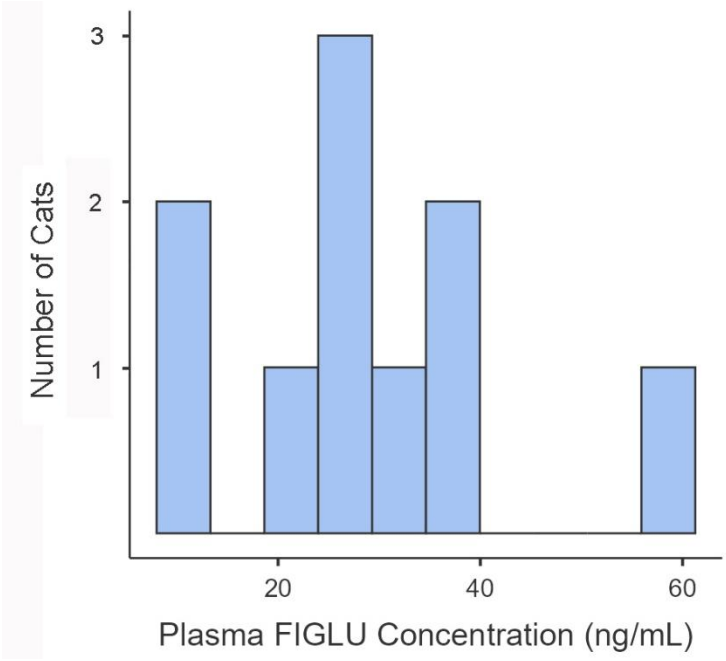


Table 10. Reference values for distributions of plasma FIGLU concentrations in groups of clinically normal cats and dogs

	Clinically normal dogs (n = 11)	**Clinically normal cats (n = 10)**
Median (ng/mL)	8.20	26.24
Minimum (ng/mL)	4.5	9.1
Maximum (ng/mL)	21.0	57.0
Interquartile range (ng/mL)	3.41	15.21
Mean (ng/mL)	9.48	28.23
Standard deviation (ng/mL)	4.93	13.76
Shapiro-Wilk W	0.845	0.95
Shapiro-Wilk *p*	0.037	0.671

3.3.3 Plasma glutamic acid concentrations in clinically normal cats and dogs

Plasma Glu concentrations ranged from 3492.5 to 8219.2 ng/mL in clinically normal dogs, and 3550.5 to 7806.0 ng/mL in clinically normal cats. All plasma Glu measurements obtained

in clinically normal cats were within a previously cited reference interval calculated from 120 clinically normal cats (2678.3 – 23 540.0 ng/mL).[227] All but one of the plasma Glu measurements obtained from clinically normal dogs fell within the range previously reported from a small group of 12 clinically normal dogs (2074.5 – 7680.2 ng/mL).[225]

3.3.4 Serum folate concentrations in clinically normal cats and dogs

Data for serum folate concentrations in clinically normal dogs and cats were not normally distributed (Figures 13 and 14, respectively). Serum folate concentrations ranged from 18.1 to > 54 nmol/L in dogs, and from 24.2 to 44.0 nmol/L in cats (Table 11). All serum folate measurements fell within the reference intervals derived from the external reference laboratory (provided in Table 11), as expected given that a population of clinically normal cats and dogs was selected for the study.[152]

Figure 13. Distribution of serum folate concentrations (nmol/L) in 11 clinically normal dogs

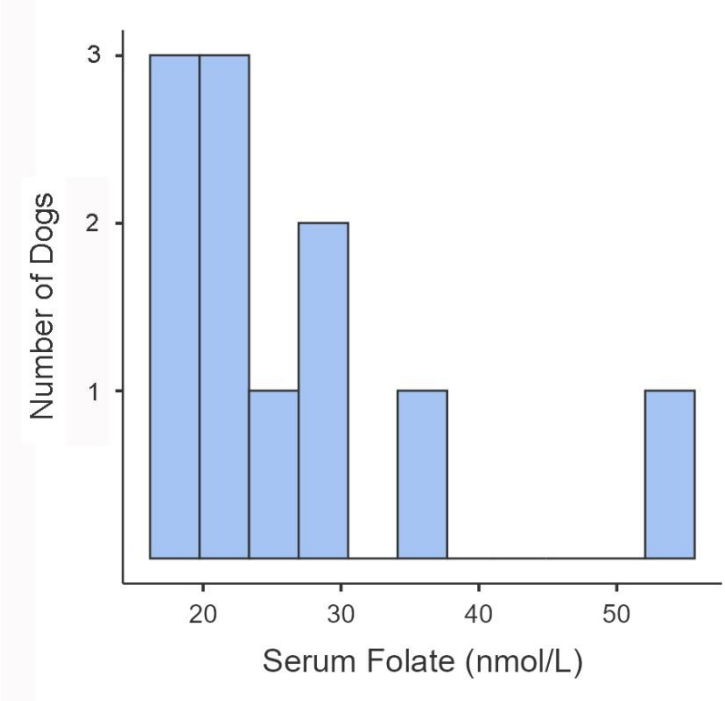


Figure 14. Distribution of serum folate concentrations (nmol/L) in 10 clinically normal cats

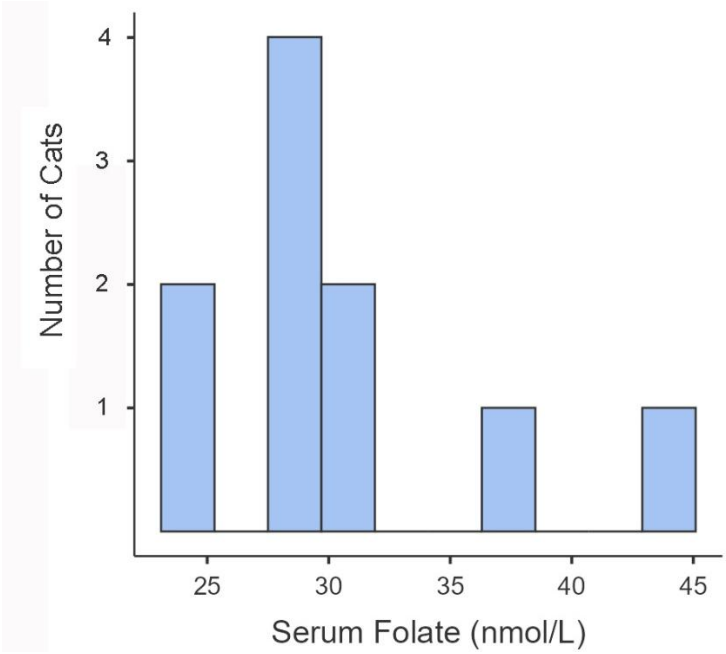


Table 11. Reference values for distributions of serum folate concentrations in groups of clinically normal cats and dogs

	Clinically normal dogs (n = 11)	**Clinically normal cats (n = 10)**
Median (nmol/L)	23.10	29.05
Minimum (nmol/L)	18.1	24.2
Maximum (nmol/L)	> 54	44.0
External laboratory reference interval[152] (nmol/L)	17.5 – 55.5	22.0 – 49.1
Interquartile range (nmol/L)	8.75	3.10
Mean (nmol/L)	26.59	30.83
Standard deviation (nmol/L)	10.53	5.98
Shapiro-Wilk W	0.768	0.859
Shapiro-Wilk *p*	0.004	0.073

3.3.5 Correlation between plasma FIGLU and serum folate concentrations in clinically normal cats and dogs

Using Spearman Rank Correlation, there was no evidence of a correlation between serum folate and plasma FIGLU concentrations in clinically normal dogs (Spearman's rho = 0.255, df = 9, P = 0.451) (Figure 15). There was also no evidence of a correlation between serum folate and plasma FIGLU concentrations in clinically normal cats based on calculation of the Spearman Rank Correlation Coefficient (Spearman's rho = -0.527, df = 8, P = 0.123) (Figure 16). A non-parametric test was selected for correlation analysis because of the use of small sample sizes and due to the distributions of plasma FIGLU and serum folate appearing skewed.

Figure 15. Scatter plot of the correlation between serum folate and plasma FIGLU concentrations in 11 clinically normal dogs

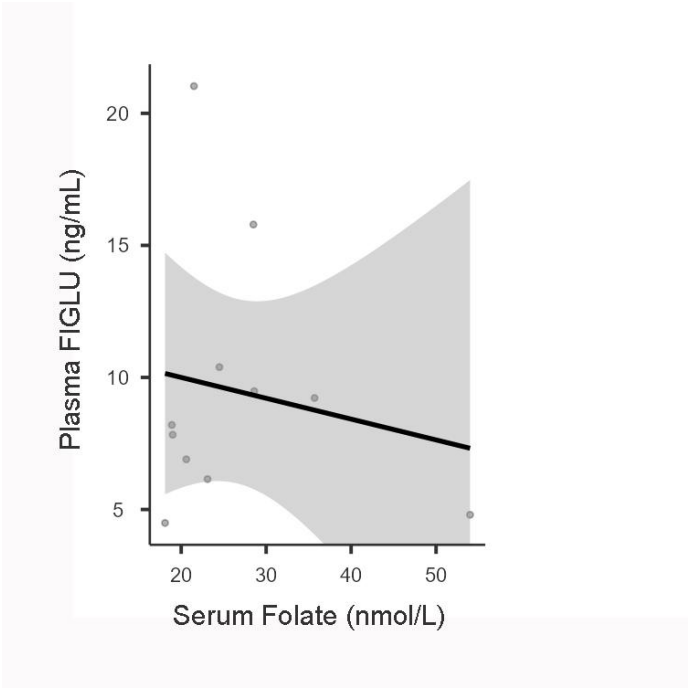
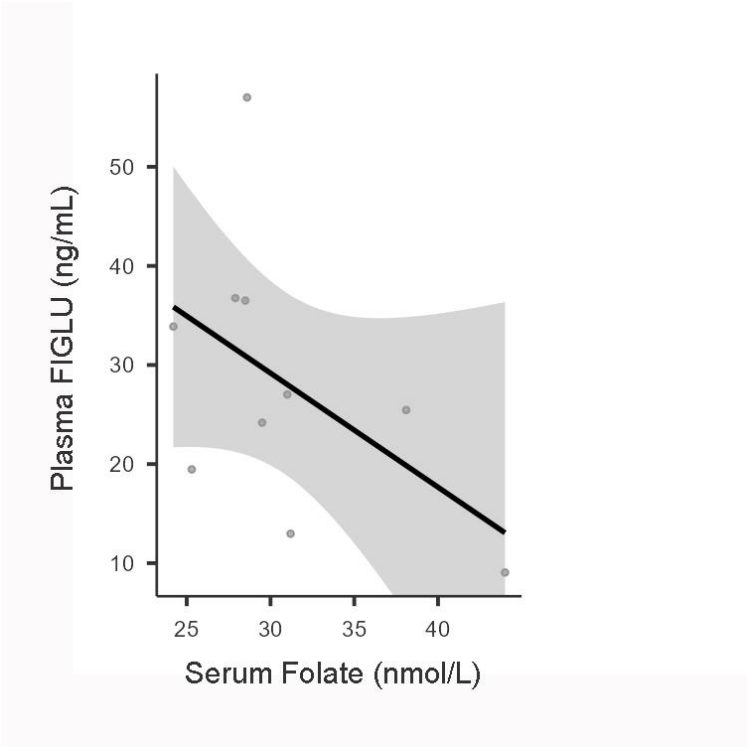


Figure 16. Scatter plot of the correlation between serum folate and plasma FIGLU concentrations in 10 clinically normal cats



3.3.6 Plasma FIGLU concentration in patient receiving long-term TMS

The dog receiving TMS demonstrated a plasma FIGLU concentration of 330.2 ng/mL, which was dramatically higher than the range observed in clinically normal dogs (4.5 - 21.0 ng/mL). The dog also displayed a plasma Glu concentration of 71 315.1 ng/mL, which was far higher than the range observed in the group of clinically normal dogs (3492.5 - 8219.2 ng/mL).

Plasma folate was within the reference interval developed for serum folate, at 30.7 nmol/L. While the accuracy of this result cannot be fully verified in an unvalidated sample type, given how far this result was from the lower reference limit (< 17.5 nmol/L), it appears unlikely that the patient had a markedly decreased blood concentration of folate.

The markedly elevated plasma FIGLU concentration combined with an apparently adequate plasma folate concentration are suggestive of a functional folate deficiency, which has been reported to be an adverse effect in dogs and humans receiving TMS.[231,236] Trimethoprim

inhibits the enzyme dihydrofolate reductase (DHFR), which converts dihydrofolate (DHF) to the biologically active form of folate, THF.[236] In spite of mammalian DHFR being 10 000 times less sensitive to trimethoprim than bacterial DHFR, trimethoprim has nevertheless been shown to affect folate utilisation in animals and humans.[236] Trimethoprim can thereby induce a functional folate deficiency, where cells are unable to utilise folate effectively even in the presence of adequate folate concentrations.[236]

In spite of this biochemical evidence of folate deficiency, a CBC performed at the time of blood collection did not demonstrate any changes suggestive of haematologic complications from folate deficiency. The dog was also clinically well, and displayed no evidence of gastrointestinal or neurologic complications of folate deficiency. In fact, while diarrhoea, inappetence and lethargy had been observed prior to starting TMS due to uncontrolled bacterial cholangitis, these clinical signs had completely resolved by the time of blood collection, presumably due to a positive response to TMS therapy.

3.4 Discussion

This is the first study to report blood FIGLU concentrations in a group of cats and dogs. The ranges of plasma FIGLU concentrations observed in clinically normal dogs and clinically normal cats respectively were 4.5 - 21.0 ng/mL, and 9.1 to 57.0 ng/mL. Given that the reference interval for plasma FIGLU has been reported in humans as 37 to 273 ng/mL, plasma FIGLU concentrations were lower in clinically normal cats and dogs, compared to healthy humans.[203] Of key importance, however, is that the amount of FIGLU present in clinically normal cats and dogs is high enough to be quantifiable via LC-MS/MS techniques, and reference interval determination of plasma FIGLU in these animal species is therefore a feasible task.

As further work on the LC-MS/MS method is needed to minimise the impact of ion suppression on plasma FIGLU results, the author decided not to analyse the large numbers of samples required to develop reference intervals. This pilot study, however, did generate some valuable data that demonstrates the potential utility of spot plasma FIGLU measurement in the evaluation of folate deficiency in cats and dogs.

Of all the samples collected during the project, only two samples demonstrated markedly higher FIGLU concentrations than the remaining samples. These included the sample collected from the dog that received TMS long-term (330.2 ng/mL), and the pool of ill canine plasma (490 ng/mL) that was utilised as a QC for method validation experiments. In retrospect, it appears likely that there was one or several dogs amongst the ill canine plasma pool that had folate deficiency and resulting marked elevations in plasma FIGLU. While one could raise the possibility of plasma FIGLU being a non-specific indicator of ill health that commonly rises in any sick animal, this appears less likely when the pool of ill feline plasma had FIGLU concentrations only slightly higher than the maximum result recorded in clinically normal cats (75 ng/mL compared to the maximum of 57 ng/mL). Furthermore, the dog that was receiving TMS was actually clinically normal at the time of blood collection, and was selected for analysis only because it was hypothesised that its folate status was likely to be altered by TMS administration. These findings support the hypothesis that plasma FIGLU rises in animals with folate deficiency, and could therefore be an effective metabolic marker of folate deficiency in cats and dogs.

One unexpected finding in this project was that all three samples obtained from ill animals demonstrated profoundly elevated plasma glutamic acid concentrations. Plasma glutamic acid concentrations were 71 315, 92 000 and 102 500 ng/mL respectively in the patient on TMS, ill canine plasma pool, and ill feline plasma pool. In contrast, plasma glutamic acid concentrations obtained in clinically normal animals using this method were very similar to

other reports. Given the results in clinically normal animals being similar to those obtained by previous methods, this suggests that this current assay is likely fairly accurate in the quantitation of plasma glutamic acid. Thus the surprising results obtained from ill patients is unlikely primarily due to accuracy errors in this assay.

A major implication of this finding is that ion suppression caused by high plasma glutamic acid concentrations is likely to be a common issue in LC-MS/MS analysis of samples obtained from ill animals. While ion suppression was observed in this project at lower glutamic acid concentrations as well, the higher glutamic acid concentrations in ill animal samples did produce far greater ion suppression, as evidenced by the poor dilution integrity experimental results when testing ill animal plasma. As the goal is to eventually test samples from ill hypofolataemic animals, which the author can now expect to commonly have markedly elevated glutamic acid concentrations, this emphasises the importance of resolving the issue of glutamic acid-induced ion suppression in this assay.

As previously discussed, use of a stable isotope labelled FIGLU standard as the IS could potentially alleviate the detrimental effects of glutamic acid-induced ion suppression on this plasma FIGLU assay. Future projects quantifying plasma FIGLU should therefore aim to accrue adequate funds to obtain this standard, and collaborate with chemists that custom produce stable isotope labelled standards.

In this current project, a correlation was not observed between plasma FIGLU and serum folate concentrations in clinically normal cats and dogs. This was not a surprising finding, because as long as healthy animals have sufficient folate levels, folate-dependent FIGLU metabolism is expected to occur at an adequate rate. Once a large number of hypofolataemic and folate-deficient animals are included in the study population, however, a correlation is expected to develop. This current pilot study did not incorporate a population of

hypofolataemic animals. The small size of the sampling population may have also contributed to the lack of detection of a correlation, by reducing the study's statistical power. While results did not reach statistical significance, the scatter plots did display a trend towards a negative correlation between plasma FIGLU and serum folate concentrations. Future studies should therefore analyse samples from a larger number of animals, including a mixture of both hypofolataemic and normofolataemic subjects. Once folate-deficient subjects are included in the statistical analysis, it is expected that correlation testing will demonstrate a strong negative correlation between plasma FIGLU and serum folate concentrations.

As serum folate measurement is an uncommon practice in small animals in Australia, largely due to its high expense, the local acquisition of large numbers of hypofolataemic samples is difficult. Collaboration with international laboratories that have a high turnover of performing folate assays in cats and dogs would be the best way to obtain large numbers of hypofolataemic patient samples. Stability studies would need to be performed, though, to confirm that plasma FIGLU is sufficiently stable to handle international refrigerated transport.

Potential effects of transport were also the reason why a non-validated folate assay was utilised in this current study. No serum folate assays are commercially available in Australia that have been analytically validated for use in cats and dogs. The option of transporting serum samples internationally, to be tested at a laboratory where the assay had been validated in animals, was heavily considered. However, serum folate has only been found to be stable stored at refrigerated temperatures for up to 24 hours and international transport from Australia is likely to exceed that time period.[144] Therefore the potential detrimental impact of transporting samples internationally was considered of greater significance than using an assay that had not been validated in cats and dogs, but that had undergone extensive validation in humans. Transporting samples frozen on dry ice would be a way to avoid these

stability concerns, and serum folate has been shown to be stable at -20°C for up to eight weeks.[144] However, the cost required for international transport of samples on dry ice exceeded the financial resources available for this project.

The author strongly recommends that analytical validation is undertaken on the folate assays commercially available in Australia for feline and canine serum. A locally-derived reference interval should also be generated for feline and canine serum folate using the Atellica IM Analyzer.

3.5 Conclusions

In conclusion, the range of plasma FIGLU concentrations observed in groups of clinically normal cats and dogs were reported herein. Larger studies with sample sizes over 40 are recommended in the future, to develop reference intervals for canine and feline plasma FIGLU concentrations. Based on data obtained in this pilot study, plasma FIGLU concentrations in clinically normal cats and dogs are high enough to be quantified via LC-MS/MS analysis, making it possible for reference intervals to be generated.

Further, a dog receiving TMS long-term with suspected functional folate deficiency demonstrated a plasma FIGLU concentration markedly higher than the range observed in clinically normal dogs. This supports the theory that plasma FIGLU measurement can be used as a functional indicator of folate deficiency in cats and dogs.

FUTURE DIRECTIONS

The ultimate goal for FIGLU research is to identify the serum folate cut-off limit at which cats and dogs start developing biochemical evidence of folate deficiency. Once future studies have analysed serum folate and plasma FIGLU concentrations in a large group of hypofolataemic and normofolataemic animals, statistical analyses can be performed to determine the optimal serum folate cut-off value below which plasma FIGLU concentrations begin to rise. This would help to establish a lower reference limit for serum folate that accurately predicts low tissue folate stores and whole-body folate deficiency in cats and dogs. Calculation of this optimal lower reference limit for serum folate will help the veterinary industry two-fold. Firstly, clinicians can confidently diagnose cats and dogs with true whole-body folate deficiency based on serum folate measurement. Secondly, refinement of the optimal serum folate lower reference limit would put researchers in a much better position to be able to select study populations of cats and dogs that have true whole-body folate deficiencies. This select population of folate-deficient cats and dogs can then be closely inspected through observational studies for evidence of haematologic or clinical complications associated with the deficiency. Further, interventional studies could be performed on folate-deficient cats and dogs to assess for clinical and haematologic responses to folic acid supplementation.

These two types of studies will help to answer some fundamental research questions that are required for clinicians to be able to take an evidence-based approach to folic acid supplementation. First, the hope is to discover whether hypofolataemia is truly of any clinical significance in cats and dogs; as in, whether folate deficiency has clinical and haematologic complications in small animals. Second, the author strives to reach an answer as to whether folic acid supplementation of folate-deficient feline and canine patients confers a clinical

benefit. Finally, if folic acid supplementation is deemed to be beneficial in deficient animals, the threshold serum folate concentration at which supplementation is warranted, should be determined. Use of FIGLU as a metabolic marker could thereby greatly expand the body of knowledge available in veterinary medicine, relating to folate deficiency in cats and dogs.

REFERENCES

1. Scaglione F, Panzavolta G. Folate, folic acid and 5-methyltetrahydrofolate are not the same thing. *Xenobiotica*. 2014;44(5):480-488. doi:10.3109/00498254.2013.845705

2. Kim Y. Folate and colorectal cancer: an evidence-based critical review. *Mol Nutr Food Res*. 2007;51(3):267-292. doi:10.1002/mnfr.200600191

3. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification-its history, effect, concerns, and future directions. *Nutrients*. 2011;3(3):370-384. doi:10.3390/nu3030370

4. Ponziani FR, Cazzato IA, Danese S, *et al*. Folate in gastrointestinal health and disease. *Eur Rev Med Pharmaco*. 2012;16(3):376-385.

5. Tran QD, Hendriks WH, van der Poel AF. Effects of extrusion processing on nutrients in dry pet food. *J Sci Food Agr*. 2008;88(9):1487-1493. doi:10.1002/jsfa.3247

6. de Giori GS, LeBlanc JG, eds. Folate production by lactic acid bacteria. In: *Polyphenols: Prevention and Treatment of Human Disease*. 2nd ed. Elsevier; 2018:15-29.

7. Pantůčková P, Křivánková L. Analysis of 5-methyltetrahydrofolate in human blood, serum and urine by on-line coupling of capillary isotachophoresis and zone electrophoresis. *Electrophoresis*. 2010;31(20):3391-3399. doi:10.1002/elps.201000193

8. Sysel AM, Horne WI, Steiner JM, Suchodolski JS, Bauer JA. Serum folate: a pharmacodynamic biomarker of intracellular nitrosylcobalamin activity following intravenous administration in dogs. *Anticancer Res*. 2012;32(10):4307-4312.

9. Sobczyńska-Malefora A, Harrington DJ, Voong K, Shearer MJ. Plasma and red cell reference intervals of 5-methyltetrahydrofolate of healthy adults in whom biochemical functional deficiencies of folate and vitamin B12 had been excluded. *Adv Hematology*. 2014;2014:465623. doi:10.1155/2014/465623

10. Pravenec M, Leung KY, Zídek V, *et al*. Genetically determined folate deficiency is associated with abnormal hepatic folate profiles in the spontaneously hypertensive rat. *Physiol Res*. 2018;67(3):417-422. doi:10.33549/physiolres.933897

11. Sobczyńska-Malefora A. Methods for assessment of folate (vitamin B9). In: Harrington D, ed. *Laboratory Assessment of Vitamin Status*. Elsevier; 2019:219-264. doi:10.1016/b978-0-12-813050-6.00011-5

12. Forssén KM, Jägerstad MI, Wigertz K, Witthöft CM. Folates and dairy products: a critical update. *J Am Coll Nutr*. 2000;19(sup2):100S-110S. doi:10.1080/07315724.2000.10718071

13. McNulty H, Pentieva K. Folate bioavailability. *P Nutr Soc*. 2004;63(4):529-536. doi:10.1079/pns2004383

14. Revuelta JL, Serrano-Amatriain C, Ledesma-Amaro R, Jiménez A. Formation of folates by microorganisms: towards the biotechnological production of this vitamin. *Appl Microbiol Biot*. 2018;102(20):8613-8620. doi:10.1007/s00253-018-9266-0

15. Mooney A. *Stability of Essential Nutrients in Pet Food Manufacturing and Storage*. Thesis. Kansas State University; 2010.

16. Fantinati M, Dufayet R, Rouch-Buck P, Priymenko N. Relationship between a plant-based 'vegan' pet food and clinical manifestation of multiple nutrient deficiencies in two cats. *J Anim Physiol An N*. 2021;105(6):1179-1191. doi:10.1111/jpn.13510

17. Council NR. *Nutrient Requirements of Dogs and Cats*. National Academies Press; 2006.

18. Dossin O. Laboratory tests for diagnosis of gastrointestinal and pancreatic diseases. *Top Companion Anim M*. 2011;26(2):86-97. doi:10.1053/j.tcam.2011.02.005

19. Semp PG. *Vegan Nutrition of Dogs and Cats*. Thesis. University of Veterinary Medicine Vienna; 2014.

20. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Rev Mol Med*. 2009;11:e4. doi:10.1017/s1462399409000969

21. Visentin M, Diop-Bove N, Zhao R, Goldman ID. The intestinal absorption of folates. *Annu Rev Physiol*. 2014;76(1):251-274. doi:10.1146/annurev-physiol-020911-153251

22. Zhao R, Min SH, Wang Y, Campanella E, Low PS, Goldman ID. A role for the proton-coupled folate transporter (PCFT-SLC46A1) in folate receptor-mediated endocytosis. *J Biol Chem*. 2009;284(7):4267-4274. doi:10.1074/jbc.m807665200

23. Slusher BS, Rojas C, Coyle JT. Glutamate carboxypeptidase II. In: Rawlings ND, Salvesen G, eds. *Handbook of Proteolytic Enzymes*. Vol 2. 3rd ed. Elsevier; 2013:1620-1627.

24. Zhao R, Aluri S, Goldman ID. The proton-coupled folate transporter (PCFT-SLC46A1) and the syndrome of systemic and cerebral folate deficiency of infancy: hereditary folate malabsorption. *Mol Aspects Med*. 2017;53:57-72. doi:10.1016/j.mam.2016.09.002

25. Patanwala I, King MJ, Barrett DA, *et al*. Folic acid handling by the human gut: implications for food fortification and supplementation. *Am J Clin Nutrition*. 2014;100(2):593-599. doi:10.3945/ajcn.113.080507

26. Kitamura Y, Kusuhara H, Sugiyama Y. Basolateral efflux mediated by multidrug resistance-associated protein 3 (MRP3/ABCC3) facilitates intestinal absorption of folates in mouse. *Pharm Res*. 2010;27(4):665-672. doi:10.1007/s11095-009-0047-4

27. Matherly LH, Hou Z. Structure and function of the reduced folate carrier a paradigm of a major facilitator superfamily mammalian nutrient transporter. *Vitam Horm*. 2008;79:145-184. doi:10.1016/s0083-6729(08)00405-6

28. da Costa M, Rothenberg SP. The interaction of folate ligands with macromolecules. In: Gordon AS, LoBue J, Silber R, eds. *The Year in Hematology*. Springer; 1977:131-151. doi:10.1007/978-1-4899-6688-9_4

29. Zettner A, Duly PE. The weak binding reaction between folate and human serum proteins. *Ann Clin Lab Sci*. 1978;8(1):57-63.

30. Holm J, Hansen SI, Lyngbye J. High-affinity binding of folate to a protein in serum of male subjects. *Clin Chim Acta*. 1980;100(2):113-119. doi:10.1016/0009-8981(80)90072-8

31. Zhao R, Goldman ID. Folate and thiamine transporters mediated by facilitative carriers (SLC19A1-3 and SLC46A1) and folate receptors. *Mol Aspects Med*. 2013;34(2-3):373-385. doi:10.1016/j.mam.2012.07.006

32. Jergens AE, Allenspach K. Feline inflammatory gastrointestinal disease. In: Little S, ed. *August's Consultations in Feline Internal Medicine, Volume 7*. Elsevier; 2016:129-137.

33. Hall EJ. Malabsorption syndromes in small animals. MSD Veterinary Manual. Updated 2024 September 2024. Accessed November 3, 2024. https://www.msdvetmanual.com/digestive-system/diseases-of-the-stomach-and-intestines-in-small-animals/malabsorption-syndromes-in-small-animals

34. Steiner JM. Laboratory evaluation of the gastrointestinal tract. In: Ettinger SJ, Feldman EC, Cote E, eds. *Textbook of Veterinary Internal Medicine - Kindle Edition*. 8th Edition. Elsevier; 2017:Kindle Locations 121373-121374.

35. Hall EJ, Simpson KW. Diseases of the small intestine. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine*. 5th Edition. WB Saunders; 2000:1182-1237.

36. Hakim NS, Walters AM, Zinsmeister AR, Sarr MG. Net absorption of water, electrolytes, glucose, and folate from the in vivo, neurally isolated canine jejunum. *Surgery*. 1992;111(4):394-401.

37. Bernstein LH, Gutstein S, Efron G, Wager G. Experimental production of elevated serum folate in dogs with intestinal blind loops: relationship of serum levels to location of the blind loop. *Gastroenterology*. 1972;63(5):815-819.

38. Sample RG, Rossi GV, Packman EW. Thiry-Vella dog as a biologic model for evaluation of drug absorption from the intestinal mucosa. *J Pharm Sci*. 1968;57(5):795-798. doi:10.1002/jps.2600570515

39. Toskes PP, Giannella RA, Jervis HR, Rout WR, Takeuchi A. Small intestinal mucosal injury in the experimental blind loop syndrome. Light- and electron-microscopic and histochemical studies. *Gastroenterology*. 1975;68(5 Pt 1):1193-1203.

40. Schmidt S, Fracasso G, Colombatti M, Naim HY. Cloning and characterization of canine prostate-specific membrane antigen. *Prostate*. 2013;73(6):642-650. doi:10.1002/pros.22605

41. Dowling M, Samuelson J, Fadl-Alla B, *et al*. Overexpression of prostate specific membrane antigen by canine hemangiosarcoma cells provides opportunity for the molecular detection of disease burdens within hemorrhagic body cavity effusions. *Plos One*. 2019;14(1):e0210297. doi:10.1371/journal.pone.0210297

42. Bernstein LH, Gutstein S, Weiner S, Efron G. The absorption and malabsorption of folic acid and its polyglutamates. *Am J Medicine*. 1970;48(5):570-579. doi:10.1016/0002-9343(70)90006-9

43. Qiu A, Min SH, Jansen M, *et al*. Rodent intestinal folate transporters (SLC46A1): secondary structure, functional properties, and response to dietary folate restriction. *Am J Physiol-cell Ph*. 2007;293(5):C1669-C1678. doi:10.1152/ajpcell.00202.2007

44. National Center for Biotechnology Information. Proton-coupled folate transporter [Felis catus]. Accession No. XP_003996543.2. Protein [Internet]. Published November 09, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/2131052821

45. National Center for Biotechnology Information. Proton-coupled folate transporter [Canis lupus familiaris]. Accession No. XP_038533383.1. Protein [Internet]. Published January 07, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_038533383.1

46. National Center for Biotechnology Information. Reduced folate transporter isoform X1 [Felis catus]. Accession No. XP_044892430.1. Protein [Internet]. Published November 09, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_044892430.1

47. National Center for Biotechnology Information. Reduced folate transporter isoform X1 [Canis lupus familiaris]. Accession No. XP_038299632.1. Protein [Internet]. Published January 07, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_038299632.1

48. Lin Y, Dueker SR, Follett JR, *et al*. Quantitation of in vivo human folate metabolism. *Am J Clin Nutrition*. 2004;80(3):680-691. doi:10.1093/ajcn/80.3.680

49. Kohlmeier M, ed. Water-soluble vitamins and nonnutrients. In: *Nutrient Metabolism*. 2nd ed. Elsevier; 2015:567-671. doi:10.1097/00006205-198109000-00008

50. Niesser M, Demmelmair H, Weith T, *et al*. Folate catabolites in spot urine as non-invasive biomarkers of folate status during habitual intake and folic acid supplementation. *Plos One*. 2013;8(2):e56194. doi:10.1371/journal.pone.0056194

51. McPartlin J, Courtney G, McNulty H, Weir D, Scott J. The quantitative analysis of endogenous folate catabolites in human urine. *Anal Biochem*. 1992;206(2):256-261. doi:10.1016/0003-2697(92)90363-c

52. Shulpekova Y, Nechaev V, Kardasheva S, *et al*. The concept of folic acid in health and disease. *Molecules*. 2021;26(12):3731. doi:10.3390/molecules26123731

53. Zhao R, Diop-Bove N, Visentin M, Goldman ID. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu Rev Nutr*. 2011;31(1):177-201. doi:10.1146/annurev-nutr-072610-145133

54. National Center for Biotechnology Information. Folate receptor alpha [*Felis catus*]. Accession No. XP_006937055.1. Protein [Internet]. Published November 09, 2021. Accessed April 17, 2025. https://www.ncbi.nlm.nih.gov/protein/XP_006937055.1

55. National Center for Biotechnology Information. Folate receptor alpha [*Canis lupus familiaris*]. Accession No. XP_038286189.1. Protein [Internet]. Published January 07, 2021. Accessed April 17, 2025. https://www.ncbi.nlm.nih.gov/protein/XP_038286189.1

56. Sadasivan E, Meng Y, Rothenberg SP. Coding sequence, genomic organization and expression of a folate binding protein gene in the rat. *Gene*. 2000;254(1-2):219-228. doi:10.1016/s0378-1119(00)00262-6

57. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. *Vitam Horm*. 2008;79:1-44. doi:10.1016/s0083-6729(08)00401-9

58. Molloy AM. Water soluble vitamins, clinical research and future application. *Subcell Biochem*. 2011;56:105-130. doi:10.1007/978-94-007-2199-9_7

59. Stover PJ. One-carbon metabolism–genome interactions in folate-associated pathologies. *J Nutrition*. 2009;139(12):2402-2405. doi:10.3945/jn.109.113670

60. Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev Cancer*. 2013;13(8):572-583. doi:10.1038/nrc3557

61. Lyon P, Strippoli V, Fang B, Cimmino L. B Vitamins and one-carbon metabolism: implications in human health and disease. *Nutrients*. 2020;12(9):2867. doi:10.3390/nu12092867

62. Appling DR. Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. *Faseb J*. 1991;5(12):2645-2651. doi:10.1096/fasebj.5.12.1916088

63. National Center for Biotechnology Information. Folylpolyglutamate synthase, mitochondrial isoform X1 [Canis lupus familiaris]. Accession No. XP_038534427.1. Protein [Internet]. Published January 07, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_038534427.1

64. National Center for Biotechnology Information. Folylpolyglutamate synthase, mitochondrial isoform X1 [Felis catus]. Accession No. XP_023098515.1. Protein [Internet]. Published November 09, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_023098515.1

65. Crider KS, Yang TP, Berry RJ, Bailey LB. Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv Nutr*. 2012;3(1):21-38. doi:10.3945/an.111.000992

66. Bailey LB, Stover PJ, McNulty H, *et al*. Biomarkers of nutrition for development-folate review. *J Nutrition*. 2015;145(7):1636S-1680S. doi:10.3945/jn.114.206599

67. Green R, Mitra AD. Megaloblastic anemias nutritional and other causes. *Med Clin N Am*. 2017;101(2):297-317. doi:10.1016/j.mcna.2016.09.013

68. Qiu A, Jansen M, Sakaris A, *et al.* Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell*. 2006;127(5):917-928. doi:10.1016/j.cell.2006.09.041

69. Bermejo F, Algaba A, Guerra I, *et al*. Should we monitor vitamin B12 and folate levels in Crohn's disease patients? *Scand J Gastroentero*. 2013;48(11):1272-1277. doi:10.3109/00365521.2013.836752

70. Yelencich E, Truong E, Widaman AM, *et al.* Avoidant restrictive food intake disorder prevalent among patients with inflammatory bowel disease. *Clin Gastroenterol H*. 2022;20(6):1282-1289. doi:10.1016/j.cgh.2021.08.009

71. Haskey N, Peña-Sánchez JN, Jones JL, Fowler SA. Development of a screening tool to detect nutrition risk in patients with inflammatory bowel disease. *Asia Pac J Clin Nutr*. 2018;27(4):756-762. doi:10.6133/apjcn.112017.01

72. Hoffbrand AV, Stewart JS, Booth CC, Mollin DL. Folate deficiency in Crohn's disease: incidence, pathogenesis, and treatment. *Brit Med J*. 1968;2(5597):71-75. doi:10.1136/bmj.2.5597.71

73. Pawson, Mehta. Review article: the diagnosis and treatment of haematinic deficiency in gastrointestinal disease. *Aliment Pharm Therap*. 1998;12(8):687-698. doi:10.1046/j.1365-2036.1998.00368.x

74. Prescott JF. Sulfonamides, diaminopyrimidines, and their combinations. In: Giguère S, Prescott JF, Dowling PM, eds. *Antimicrobial Therapy in Veterinary Medicine*. 5th Edition. John Wiley & Sons; 2013:279-294.

75. Berghoff N, Steiner JM. Laboratory tests for the diagnosis and management of chronic canine and feline enteropathies. *Vet Clin North Am Small Animal Pract*. 2011;41(2):311-328. doi:10.1016/j.cvsm.2011.01.001

76. Gastrointestinal Laboratory. Folate information. Texas A&M University Veterinary Medicine & Biomedical Sciences. Accessed April 16, 2025. https://vetmed.tamu.edu/gilab/research/folate-information/

77. Moser K, Mitze S, Teske E, von Bomhard W, Stockhaus C. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. *Tierarztl Prax Ausg K Kleintiere Heimtiere*. 2018;46(01):15-20. doi:10.15654/tpk-170445

78. Evans SE, Bonczynski JJ, Broussard JD, Han E, Baer KE. Comparison of endoscopic and full-thickness biopsy specimens for diagnosis of inflammatory bowel disease and alimentary tract lymphoma in cats. *J Am Vet Med Assoc*. 2006;229(9):1447-1450. doi:10.2460/javma.229.9.1447

79. Allenspach K, Mochel JP. Current diagnostics for chronic enteropathies in dogs. *Vet Clin Pathol*. 2022;50(S1):18-28. doi:10.1111/vcp.13068

80. Zoia A, Morris JS, Drigo M, Ventura L, McBrearty AR. Low serum folate concentrations in dogs with non-associative immune-mediated haemolytic anaemia. *Vet Rec*. 2022;190(7):e946. doi:10.1002/vetr.946

81. Seim-Wikse T, Skancke E, Nødtvedt A, *et al*. Comparison of body condition score and other minimally invasive biomarkers between dogs with gastric carcinoma and dogs with chronic gastritis. *J Am Vet Med Assoc*. 2019;254(2):226-235. doi:10.2460/javma.254.2.226

82. Galler A, Tran JL, Krammer-Lukas S, *et al*. Blood vitamin levels in dogs with chronic kidney disease. *Vet J*. 2012;192(2):226-231. doi:10.1016/j.tvjl.2011.06.026

83. Anturaniemi J, Zaldívar-López S, Moore R, *et al*. The effect of a raw vs dry diet on serum biochemical, hematologic, blood iron, B12, and folate levels in Staffordshire Bull Terriers. *Vet Clin Path*. 2020;49(2):258-269. doi:10.1111/vcp.12852

84. Edward JH. Clinical laboratory evaluation of small intestinal function. *Vet Clin North Am Small Animal Pract*. 1999;29(2):441-469. doi:10.1016/s0195-5616(99)50030-2

85. Steiner JM. Review of commonly used clinical pathology parameters for general gastrointestinal disease with emphasis on small animals. *Toxicol Pathol*. 2014;42(1):189-194.

86. Ruaux CG. Laboratory tests for the diagnosis of intestinal disorders. In: Steiner JM, ed. *Small Animal Gastroenterology*. Schlütersche; 2007:50-55.

87. Rosenberg IH. Folate absorption and malabsorption. *N Engl J Med*. 1975;293(25):1303-1308. doi:10.1056/nejm197512182932509

88. Rosenberg IH, Godwin HA, Russell RM, Franklin JL. Assessment of folate malabsorption in celiac sprue comparing synthetic [H-3] pteroylpolyglutamate ([H-3] PteGlu7) and [H-3] pteroylmonoglutamate ([H-3] PteGlu). *J Clin Inves*. 1974;53:66a.

89. Hoffbrand AV, Douglas AP, Fry L, Stewart JS. Malabsorption of dietary folate (pteroylpolyglutamates) in adult coeliac disease and dermatitis herpetiformis. *Brit Med J*. 1970;4(5727):85-89. doi:10.1136/bmj.4.5727.85

90. Halsted CH, Reisenauer AM, Romero JJ, Cantor DS, Ruebner B. Jejunal perfusion of simple and conjugated folates in celiac sprue. *J Clin Invest*. 1977;59(5):933-940. doi:10.1172/jci108715

91. Halsted CH, Reisenauer AM, Shane B, Tamura T. Availability of monoglutamyl and polyglutamyl folates in normal subjects and in patients with coeliac sprue. *Gut*. 1978;19(10):886-891. doi:10.1136/gut.19.10.886

92. Harrington D. Methods for assessment of Vitamin B12. In: Harrington DJ, ed. *Laboratory Assessment of Vitamin Status*. Elsevier; 2018:265-299. doi:10.1016/b978-0-12-813050-6.00012-7

93. Green R, Graff JP. Megaloblastic anemia. In: Teruya-Feldstein J, Kremyanskaya M, eds. *Atlas of Diagnostic Hematology*. Elsevier; 2020:47-51.

94. Naeim F, Rao PN, Song SX, Grody WW. Disorders of Red Blood Cells – Anemias. In: *Atlas of Hematopathology: Morphology, Immunophenotype, Cytogenetics, and Molecular Approaches*. Elsevier; 2013:675-704.

95. Stabler SP. Clinical folate deficiency. In: Bailey LB, ed. *Folate in Health and Disease*. Taylor & Francis Group; 2010:409-428.

96. Green R, Miller JW. Folate deficiency beyond megaloblastic anemia: hyperhomocysteinemia and other manifestations of dysfunctional folate status. *Semin Hematol*. 1999;36(1):47-64.

97. Quesenberry M, Huang A, Schiffman FJ. The clinical approach to the patient with anemia. In: Benz EJ, Jr., Berliner N, Schiffman FJ, eds. Anemia Pathophysiology, Diagnosis, and Management. Cambridge University Press; 2017:23-33. doi:10.1017/9781108586900.005

98. Devalia V, Hamilton MS, Molloy AM. Guidelines for the diagnosis and treatment of cobalamin and folate disorders. *Brit J Haematol*. 2014;166(4):496-513. doi:10.1111/bjh.12959

99. Porter SR, Scully C, Flint S. Hematologic status in recurrent aphthous stomatitis compared with other oral disease. *Oral Surg Oral Med Oral Pathol*. 1988;66(1):41-44. doi:10.1016/0030-4220(88)90064-3

100. Tyldesley WR. Stomatitis and recurrent oral ulceration: is a full blood screen necessary? *Brit J Oral Surg*. 1983;21(1):27-30. doi:10.1016/0007-117x(83)90027-6

101. Kozlak ST, Walsh SJ, Lalla RV. Reduced dietary intake of vitamin B12 and folate in patients with recurrent aphthous stomatitis. *J Oral Pathol Med*. 2010;39(5):420-423. doi:10.1111/j.1600-0714.2009.00867.x

102. Pandarathodiyil AK, Anil S, Vijayan SP. Angular cheilitis - an updated overview of the etiology, diagnosis, and management. *Int J Dentistry Oral Sci*. 2021;8(2):1433-1438. doi:10.19070/2377-8075-21000317

103. Chiang CP, Chang JYF, Wang YP, Wu YH, Wu YC, Sun A. Atrophic glossitis: etiology, serum autoantibodies, anemia, hematinic deficiencies, hyperhomocysteinemia, and management. *J Formos Med Assoc*. 2020;119(4):774-780. doi:10.1016/j.jfma.2019.04.015

104. Reynolds EH. The neurology of folic acid deficiency. *Handb Clin Neurology*. 2014;120:927-943. doi:10.1016/b978-0-7020-4087-0.00061-9

105. Santhosh-Kumar CR, Hassell KL, Deutsch JC, Kolhouse JF. Are neuropsychiatric manifestations of folate, cobalamin and pyridoxine deficiency mediated through imbalances in excitatory sulfur amino acids? *Med Hypotheses*. 1994;43(4):239-244. doi:10.1016/0306-9877(94)90073-6

106. Herbert V. Experimental nutritional folate deficiency in man. *Trans Assoc Am Physicians*. 1962;75:307-320.

107. Lökk J. News and views on folate and elderly persons. *Journals Gerontology Ser*. 2003;58(4):M354-M361. doi:10.1093/gerona/58.4.m354

108. Zhang D, Wen X, Wu W, Guo Y, Cui W. Elevated homocysteine level and folate deficiency associated with increased overall risk of carcinogenesis: meta-analysis of 83 case-control studies involving 35,758 individuals. *Plos One*. 2015;10(5):e0123423. doi:10.1371/journal.pone.0123423

109. Pieroth R, Paver S, Day S, Lammersfeld C. Folate and its impact on cancer risk. *Curr Nutrition Reports*. 2018;7(3):70-84. doi:10.1007/s13668-018-0237-y

110. Klipstein FA, Lipton SD, Schenk EA. Folate deficiency of the intestinal mucosa. *Am J Clin Nutrition*. 1973;26(7):728-737. doi:10.1093/ajcn/26.7.728

111. Davidson GP, Townley RRW. Structural and functional abnormalities of the small intestine due to nutritional folic acid deficiency in infancy. *J Pediatrics*. 1977;90(4):590-594. doi:10.1016/s0022-3476(77)80372-7

112. Bianchi A, Chipman DW, Dreskin A, Rosensweig NS. Nutritional folic acid deficiency with megaloblastic changes in the small-bowel epithelium. *New Engl J Medicine*. 1970;282(15):859-861. doi:10.1056/nejm197004092821510

113. Whitehead N, Reyner F, Lindenbaum J. Megaloblastic changes in the cervical epithelium. Association with oral contraceptive therapy and reversal with folic acid. *Nutr Rev*. 1989;47(10):318-321. doi:10.1111/j.1753-4887.1989.tb02756.x

114. Boddington MM, Spriggs AI. The epithelial cells in megaloblastic anaemias. *J Clin Pathol*. 1959;12(3):228-234. doi:10.1136/jcp.12.3.228

115. Hermos JA, Adams WH, Liu YK, Sullivan LW, Trier JS. Mucosa of the small intestine in folate-deficient alcoholics. *Ann Intern Med*. 1972;76(6):957-965. doi:10.7326/0003-4819-76-6-957

116. Winawer SJ, Sullivan LW, Herbert V, Zamcheck N. The jejunal mucosa in patients with nutritional folate deficiency and megaloblastic anemia. *New Engl J Medicine*. 1965;272(17):892-895. doi:10.1056/nejm196504292721705

117. Gough KR, Read AE, McCarthy CF, Waters AH. Megaloblastic anaemia due to nutritional deficiency of folic acid. *Q J Medicine*. 1963;32:243-256.

118. Hurdle A. Folic acid (3HFA) absorption and jejunal biopsy in mild nutritional folic acid deficiency. *Pathology*. 1970;2(3):193-198. doi:10.3109/00313027009081207

119. Batt RM, Morgan JO. Role of serum folate and vitamin B12 concentrations in the differentiation of small intestinal abnormalities in the dog. *Res Vet Sci*. 1982;32(1):17-22.

120. Ruaux CG, Steiner JM, Williams DA. Early biochemical and clinical responses to cobalamin supplementation in cats with signs of gastrointestinal disease and severe hypocobalaminemia. *J Vet Intern Med*. 2005;19(2):155-160. doi:10.1111/j.1939-1676.2005.tb02676.x

121. Kempf J, Hersberger M, Melliger RH, Reusch CE, Kook PH. Effects of 6 weeks of parenteral cobalamin supplementation on clinical and biochemical variables in cats with gastrointestinal disease. *J Vet Intern Med*. 2017;31(6):1664-1672. doi:10.1111/jvim.14830

122. Kook PH, Melliger RH, Hersberger M. Efficacy of intramuscular hydroxocobalamin supplementation in cats with cobalamin deficiency and gastrointestinal disease. *J Vet Intern Med*. 2020;34(5):1872-1878. doi:10.1111/jvim.15865

123. Toresson L, Steiner JM, Razdan P, *et al*. Comparison of efficacy of oral and parenteral cobalamin supplementation in normalising low cobalamin concentrations in dogs: a randomised controlled study. *Vet J*. 2018;232:27-32. doi:10.1016/j.tvjl.2017.12.010

124. Thenen SW, Rasmussen KM. Megaloblastic erythropoiesis and tissue depletion of folic acid in the cat. *Am J Vet Res*. 1978;39(7):1205-1207.

125. da Silva AC, de Angelis RC, Pontes MA, Guérios MM. The domestic cat as a laboratory animal for experimental nutrition studies. IV. Folic acid deficiency. *J Nutr*. 1955;56(2):199-213. doi:10.1093/jn/67.4.537

126. Afonsky D. Folic acid deficiency in the dog. *Science*. 1954;120(3124):803-805. doi:10.1126/science.120.3124.803

127. Ginoudis A, Ioannidou AM, Pardali D, Tsikna A, Polizopoulou Z. Correlation of red blood cell morphology with serum cobalamin and folate concentration in dogs with chronic diarrhea: a retrospective study. *Metabolites*. 2024;14(12):657. doi:10.3390/metabo14120657

128. Stanley E, Appleman E, Schlag A, Siegel A. Relationship between cobalamin and folate deficiencies and anemia in dogs. *J Vet Intern Med*. 2019;33(1):106-113. doi:10.1111/jvim.15348

129. Reed N, Gunn-Moore D, Simpson K. Cobalamin, folate and inorganic phosphate abnormalities in ill cats. *J Feline Med Surg*. 2007;9(4):278-288. doi:10.1016/j.jfms.2007.01.005

130. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268. doi:10.1182/blood-2004-05-1812

131. Cusick SE, Mei Z, Freedman DS, *et al.* Unexplained decline in the prevalence of anemia among US children and women between 1988–1994 and 1999–2002. *Am J Clin Nutrition*. 2008;88(6):1611-1617. doi:10.3945/ajcn.2008.25926

132. Olivares M, Hertrampf E, Capurro M, Wegner D. Prevalence of anemia in elderly subjects living at home: role of micronutrient deficiency and inflammation. *Eur J Clin Nutr*. 2000;54(11):834-839. doi:10.1038/sj.ejcn.1601099

133. Read AE, Gough KR, Pardoe JL, Nicholas A. Nutritional studies on the entrants to an old people's home, with particular reference to folic-acid deficiency. *Brit Med J*. 1965;2:843-848. doi:10.1136/bmj.2.5466.843

134. Charlton K, Kruger M, Labadarios D, Wolmarans P, Aronson I. Iron, folate and vitamin B12 status of an elderly South African population. *Eur J Clin Nutr*. 1997;51(7):424-430. doi:10.1038/sj.ejcn.1600402

135. Dupont WD, Plummer WD. Power and sample size calculations A review and computer program. *Control Clin Trials*. 1990;11(2):116-128. doi:10.1016/0197-2456(90)90005-m

136. The Royal College of Pathologists of Australasia. Folate. RCPA Manual. Updated February 6, 2024. Accessed January 16, 2025. https://www.rcpa.edu.au/Manuals/RCPA-Manual/Pathology-Tests/F/Folate

137. da Silva AC. The domestic cat as a laboratory animal for experimental nutrition studies. II. Comparative growth rate and hematology on stock and purified rations. *Acta Physiologica Latino Am*. 1950;1(1):26-32.

138. Golding PH. Severe experimental folate deficiency in a human subject- a longitudinal study of biochemical and haematological responses as megaloblastic anaemia develops. *Springerplus*. 2014;3(1):442. doi:10.1186/2193-1801-3-442

139. Gibson RS. Assessment of the status of folate and vitamin B12. In: Gibson RS, ed. *Principles of Nutritional Assessment*. Oxford University Press; 1990:461-486.

140. Antony AC. Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests. *Nutr Rev*. 2017;75(8):600-615. doi:10.1093/nutrit/nux032

141. Wu A, Chanarin I, Slavin G, Levi AJ. Folate deficiency in the alcoholic—its relationship to clinical and haematological abnormalities, liver disease and folate stores. *Brit J Haematol*. 1975;29(3):469-478. doi:10.1111/j.1365-2141.1975.tb01844.x

142. World Health Organization. Serum and red blood cell folate concentrations for assessing folate status in populations. Vitamin and Mineral Nutrition Information System. Published 2015. Accessed April 17, 2025. http://apps.who.int/iris/bitstream/10665/162114/1/WHO_NMH_NHD_EPG_15.01.pdf?ua=1

143. Sobczyńska-Malefora A, Harrington DJ. Laboratory assessment of folate (vitamin B9) status. *J Clin Pathol*. 2018;71(11):949. doi:10.1136/jclinpath-2018-205048

144. World Health Organization Use of anticoagulants in diagnostic laboratory investigations. Published 2002. Accessed April 17, 2025. https://apps.who.int/iris/bitstream/handle/10665/65957/WHO_DIL_LAB_99.1_REV.2.pdf

145. Gilfix BM. Utility of measuring serum or red blood cell folate in the era of folate fortification of flour. *Clin Biochem*. 2014;47(7-8):533-538. doi:10.1016/j.clinbiochem.2014.01.023

146. Farrell CJL, Kirsch SH, Herrmann M. Red cell or serum folate: what to do in clinical practice? *Clin Chem Lab Med*. 2013;51(3):555-569. doi:10.1515/cclm-2012-0639

147. Amos RJ, Dawson DW, Fish DI, Leeming RJ, Linnell JC. Guidelines on the investigation and diagnosis of cobalamin and folate deficiencies. *Clin Laboratory Haematol*. 1994;16(2):101-115. doi:10.1111/j.1365-2257.1994.tb00397.x

148. Galloway M, Rushworth L. Red cell or serum folate? Results from the National Pathology Alliance benchmarking review. *J Clin Pathol*. 2003;56(12):924-926. doi:10.1136/jcp.56.12.924

149. Shane B. Folate status assessment history: implications for measurement of biomarkers in NHANES. *Am J Clin Nutrition*. 2011;94(1):337S-342S. doi:10.3945/ajcn.111.013367

150. Batt RM, McLean L, Rutgers HC, Hall EJ. Validation of a radioassay for the determination of serum folate and cobalamin concentrations in dogs. *J Small Anim Pract*. 1991;32(5):221-224. doi:10.1111/j.1748-5827.1991.tb00552.x

151. McLeish SA, Burt K, Papasouliotis K. Analytical quality assessment and method comparison of immunoassays for the measurement of serum cobalamin and folate in dogs and cats. *J Vet Diagn Invest*. 2019;31(2):164-174. doi:10.1177/1040638718824073

152. Gastrointestinal Laboratory. Serum cobalamin (vitamin B12) and folate. Texas A&M University Veterinary Medicine & Biomedical Sciences. Accessed April 16, 2025. https://vetmed.tamu.edu/gilab/service/assays/b12folate/

153. Amyes SJ, Roberts PM, Scott PP, Suri BS. Post-natal changes in red cell, plasma and liver folate concentrations of the cat, rat, rabbit and dog. *J Physiology*. 1973;232(1):23P-24P.

154. Switzer BR, Anderson JJB, Pick JR. Effects of dietary protein and ethanol intake on pregnant beagles fed purified diets. *J Nutrition*. 1986;116(4):689-697. doi:10.1093/jn/116.4.689

155. Bunch SE, Easley JR, Cullen JM. Hematologic values and plasma and tissue folate concentrations in dogs given phenytoin on a long-term basis. *Am J Vet Res*. 1990;51(11):1865-1868.

156. WHO Scientific Group on Nutritional Anaemias & World Health Organization. Nutritional anaemias: report of a WHO scientific group. Published 1968. Accessed April 17, 2025. https://iris.who.int/handle/10665/40707

157. de Benoist B. Conclusions of a WHO technical consultation on folate and vitamin B12 deficiencies. *Food Nutr Bull*. 2008;29(2 Suppl.):S238–44. doi:10.1177/15648265080292s129

158. Selhub J, Jacques PF, Dallal G, Choumenkovitch S, Rogers G. The use of blood concentrations of vitamins and their respective functional indicators to define folate and vitamin B12 status. *Food Nutr Bull*. 2008;29(2_suppl1):S67-S73. doi:10.1177/15648265080292s110

159. Pfeiffer CM, Sternberg MR, Hamner HC, *et al.* Applying inappropriate cutoffs leads to misinterpretation of folate status in the US population. *Am J Clin Nutrition*. 2016;104(6):1607-1615. doi:10.3945/ajcn.116.138529

160. Dickey W, Ward M, Whittle CR, *et al.* Homocysteine and related B-vitamin status in coeliac disease: effects of gluten exclusion and histological recovery. *Scand J Gastroentero*. 2009;43(6):682-688. doi:10.1080/00365520701881118

161. Monsen ALB, Ueland PM. Homocysteine and methylmalonic acid in diagnosis and risk assessment from infancy to adolescence. *Am J Clin Nutrition*. 2003;78(1):7-21. doi:10.1093/ajcn/78.1.7

162. Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. *Am J Medicine*. 1994;96(3):239-246. doi:10.1016/0002-9343(94)90149-x

163. Benvenuti E, Pierini A, Gori E, *et al*. Serum homocysteine concentration in dogs with immunosuppressant-responsive enteropathy. *J Vet Sci*. 2020;21(4):e47. doi:10.4142/jvs.2020.21.e47

164. Heilmann RM, Grützner N, Iazbik MC, *et al*. Hyperhomocysteinemia in greyhounds and its association with hypofolatemia and other clinicopathologic variables. *J Vet Intern Med*. 2017;31(1):109-116. doi:10.1111/jvim.14597

165. Johnson KL, Tiedeman T, Peterson H, Steiner JM, Trepanier LA. Potential mechanism for hyperhomocysteinemia in Greyhound dogs. *J Vet Intern Med*. 2023;37(3):960-967. doi:10.1111/jvim.16700

166. Ruaux CG, Steiner JM, Williams DA. Metabolism of amino acids in cats with severe cobalamin deficiency. *Am J Vet Res*. 2001;62(12):1852-1858. doi:10.2460/ajvr.2001.62.1852

167. Finkelstein JD. The metabolism of homocysteine: pathways and regulation. *Eur J Pediatr*. 1998;157(Suppl 2):S40-S44. doi:10.1007/pl00014300

168. Ulvik A, Theofylaktopoulou D, Midttun Ø, Nygård O, Eussen SJ, Ueland PM. Substrate product ratios of enzymes in the kynurenine pathway measured in plasma as indicators of functional vitamin B-6 status. *Am J Clin Nutr*. 2013;98(4):934-940. doi:10.3945/ajcn.113.064998

169. Ulvik A, Hustad S, McCann A, Midttun Ø, Nygård OK, Ueland PM. Ratios of one-carbon metabolites are functional markers of b-vitamin status in a norwegian coronary angiography screening cohort. *J Nutr*. 2017;147(6):1167-1173. doi:10.3945/jn.116.244657

170. Gylling B, Myte R, Ulvik A, *et al*. One-carbon metabolite ratios as functional B-vitamin markers and in relation to colorectal cancer risk. *Int J Cancer*. 2019;144(5):947-956. doi:10.1002/ijc.31606

171. Heilmann RM, Berghoff N, Mansell J, *et al*. Association of fecal calprotectin concentrations with disease severity, response to treatment, and other biomarkers in dogs with chronic inflammatory enteropathies. *J Vet Intern Med*. 2018;32(2):679-692. doi:10.1111/jvim.15065

172. Petrelli A, Salavati S. Retrospective analysis of prevalence and clinical significance of hypo- or hyperfolataemia in dogs with chronic enteropathies [ECVIM abstract ESCG-O-5]. *J Vet Intern Med*. 2020;34(1):341.

173. Dandrieux JRS, Noble PJM, Halladay LJ, McLean L, German AJ. Canine breed predispositions for marked hypocobalaminaemia or decreased folate concentration assessed by a laboratory survey. *J Small Anim Pract*. 2013;54(3):143-148. doi:10.1111/jsap.12039

174. Kathrani A, Fascetti AJ, Larsen JA, Maunder C, Hall EJ. Whole-blood taurine concentrations in cats with intestinal disease. *J Vet Intern Med*. 2017;31(4):1067-1073. doi:10.1111/jvim.14773

175. Burke KF, Broussard JD, Ruaux CG, Suchodolski JS, Williams DA, Steiner JM. Evaluation of fecal α1-proteinase inhibitor concentrations in cats with idiopathic inflammatory bowel disease and cats with gastrointestinal neoplasia. *Vet J*. 2013;196(2):189-196. doi:10.1016/j.tvjl.2012.09.019

176. Simpson KW, Miller ML, Loftus JP, Rishniw M, Frederick CE, Wakshlag JJ. Randomized controlled trial of hydrolyzed fish diets in dogs with chronic enteropathy. *J Vet Intern Med*. 2023;37(6):2334-2343. doi:10.1111/jvim.16844

177. Heilmann RM, Steiner JM. Clinical utility of currently available biomarkers in inflammatory enteropathies of dogs. *J Vet Intern Med*. 2018;32(5):1495-1508. doi:10.1111/jvim.15247

178. Plumb DC. Folic acid. Plumb's Veterinary Drugs. Updated April, 2022. Accessed April 17, 2025. https://app.plumbs.com/drug-monograph/9s847s2wkAPROD

179. Kim Y. Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. *Environ Mol Mutagen*. 2004;44(1):10-25. doi:10.1002/em.20025

180. European Food Safety Authority (EFSA). ESCO report on analysis of risks and benefits of fortification of food with folic acid. *EFSA Supporting Publ.* 2009;6(8). doi:10.2903/sp.efsa.2009.en-3

181. Clarke R, Halsey J, Lewington S, *et al*. Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: meta-analysis of 8 randomized trials involving 37 485 individuals. *Arch Intern Med*. 2010;170(18):1622-1631. doi:10.1001/archinternmed.2010.348

182. Haam JH, Lee YK, Suh E, Choi SW, Chun H, Kim YS. Urine organic acids may be useful biomarkers for metabolic syndrome and its components in Korean adults. *Clin Chem Lab Med (CCLM)*. 2021;59(11):1824-1831. doi:10.1515/cclm-2021-0598

183. Lascelles PT, Donaldson D. Formiminoglutamic acid (FIGLU) test. In: *Diagnostic Function Tests in Chemical Pathology*. Springer; 1989:59-60. doi:10.1007/978-94-009-1846-7_31

184. Holeček M. Histidine in health and disease: metabolism, physiological importance, and use as a supplement. *Nutrients*. 2020;12(3):848. doi:10.3390/nu12030848

185. Brosnan ME, Brosnan JT. Histidine metabolism and function. *J Nutr*. 2020;150(Suppl 1):2570S-2575S. doi:10.1093/jn/nxaa079

186. Kohls D, Sulea T, Purisima EO, MacKenzie RE, Vrielink A. The crystal structure of the formiminotransferase domain of formiminotransferase-cyclodeaminase: implications for substrate channeling in a bifunctional enzyme. *Structure*. 2000;8(1):35-46. doi:10.1016/s0969-2126(00)00078-2

187. Tabor H, Wyngarden L. A method for the determination of formiminoglutamic acid in urine. *J Clin Invest*. 1958;37(6):824-828. doi:10.1172/jci103670

188. Knowles J, Shaldon S, Fleming A. Folic acid metabolism in liver disease. *Clin Sci*. 1963;24:29-45.

189. Bashour A, Bloom G. 58K, a Microtubule-binding Golgi protein, is a formiminotransferase cyclodeaminase. *J Biol Chem*. 1998;273:19612–19617.

190. Hennig D, Scales S, Moreau A, Murley L, Mey JD, Kreis T. A formiminotransferase cyclodeaminase isoform is localized to the Golgi complex and can mediate interaction of trans-Golgi network-derived vesicles with microtubules. *J Biol Chem*. 1998;273(31):19602-19611. doi:10.1074/jbc.273.31.19602

191. National Center for Biotechnology Information. Formimidoyltransferase-cyclodeaminase isoform X1 [Felis catus]. Accession No. XP_011284017.2. Protein [Internet]. Published November 09, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_011284017.2

192. National Center for Biotechnology Information. Formimidoyltransferase-cyclodeaminase isoform X1 [Canis lupus familiaris]. Accession No. XP_038437080.1. Protein [Internet]. Published January 06, 2021. Accessed June 18, 2024. https://www.ncbi.nlm.nih.gov/protein/XP_038437080.1

193. Kim S, Chen J, Cheng T. PubChem in 2021: new data content and improved web interfaces. *Nucleic Acids Res*. 2021;49(D1):D1388–D1395. doi:10.1093/nar/gkaa971

194. Solans A, Estivill X, de la Luna S. Cloning and characterization of human FTCD on 21q22.3, a candidate gene for glutamate formiminotransferase deficiency. *Cytogenet Genome Res*. 2000;88(1-2):43-49. doi:10.1159/000015483

195. Luhby AL, Cooperman JM, Teller DN. Urinary excretion of formiminoglutamic acid. *Am J Clin Nutrition*. 1959;7(4):397-406. doi:10.1093/ajcn/7.4.397

196. Yu S, Morris JG. Folate requirement of growing kittens to prevent elevated formiminoglutamic acid excretion following histidine loading. *J Nutrition*. 1998;128(12):2606S-2608S. doi:10.1093/jn/128.12.2606s

197. Chanarin I, Hutchinson M, McLean A, Moule M. Hepatic folate in man. *Brit Med J*. 1966;1(5484):396-399. doi:10.1136/bmj.1.5484.396

198. Ryder RJW, Path MC. An evaluation of the measurement of urinary formiminoglutamic acid excretion as an indication of disturbed folic acid metabolism. *Ir J Medical Sci*. 1964;39(9):403-411. doi:10.1007/bf02944738

199. Chapman MJ, Wallace EC, Pollock TA. Organic acid profiling. In: Pizzorno J, Murray M, eds. *Textbook of Natural Medicine 5th Edition*. Elsevier; 2020:236-244. doi:10.1016/b978-0-323-43044-9.00029-7

200. Kohn J, Mollin DL, Rosenbach LM. Conventional voltage electrophoresis for formiminoglutamic-acid determination in folic acid deficiency. *J Clin Pathol*. 1961;14(4):345-350. doi:10.1136/jcp.14.4.345

201. Duran M, Ketting D, de Bree P, *et al*. A case of formiminoglutamic aciduria. *Eur J Pediatr*. 1981;136(3):319-323. doi:10.1007/bf00443002

202. Perry T, Applegarth D, Evans M, Hansen S, Jellum E. Metabolic studies of a family with massive formiminoglutamic aciduria. *Pediatr Res*. 1975;9(3):117-122. doi:10.1203/00006450-197503000-00001

203. Malvagia S, la Marca G, Casetta B, *et al*. Falsely elevated C4-carnitine as expression of glutamate formiminotransferase deficiency in tandem mass spectrometry newborn screening [Letter to the Editor]. *J Mass Spectrom*. 2006;41(2):263-265. doi:10.1002/jms.964

204. Weiner I, Mudge G. Renal tubular mechanisms for excretion of organic acids and bases. *Am J Med*. 1964;36:743-762.

205. Marshall R, Jandl J. Responses to “physiologic” doses of folic acid in megaloblastic anemias. *Arch Intern Med*. 1960;105(3):352-360. doi:10.1001/archinte.1960.00270150006002

206. Ahrens-Nicklas RC, Ganetzky RD, Rush PW, Conway RL, Ficicioglu C. Characteristics and outcomes of patients with formiminoglutamic aciduria detected through newborn screening. *J Inherit Metab Dis*. 2019;42(1):140-146. doi:10.1002/jimd.12035

207. Majumdar R, Yori A, Rush PW, *et al*. Allelic spectrum of formiminotransferase-cyclodeaminase gene variants in individuals with formiminoglutamic aciduria. *Mol Genet Genomic Med*. 2017;5(6):795-799. doi:10.1002/mgg3.333

208. Silverman M, Pitney A. Dietary methionine and the excretion of formiminoglutamic acid by the rat. *J Biol Chem*. 1958;233(5):1179-1182.

209. Zalusky R, Herbert V. Failure of formiminoglutamic acid (FiGlu) excretion to distinguish vitamin B12 deficiency from nutritional folic acid deficiency. *Journal Clin Investig*. 1961;40(6):1091.

210. Merritt A, Rucknagel D, Silverman M, Gardiner R. Urinary urocanic acid in man: the identification of urocanic acid and the comparative excretions of urocanic acid and n-formiminoglutamic acid after oral histidine in patients with liver disease. *J Clin Investig*. 1962;41(7):1472-1483. doi:10.1172/jci104602

211. Luhby A, Cooperman JM, Teller D. Histidine metabolic loading test to distinguish folic acid deficiency from vit. B12 in megaloblastic anemias. *Proc Soc Exp Biol Med*. 1959;101(2):350-352. doi:10.3181/00379727-101-24936

212. Spray G, Witts L. Excretion of formiminoglutamic acid as an index of folic-acid deficiency. *Lancet*. 1959;2(7105):702-704. doi:10.1016/s0140-6736(59)92132-4

213. Marín G, Tentoni J, Cicchetti G. Megaloblastic anemia- rapid and economical study. *Sangre (Barc)*. 1997;42(3):235-238.

214. Shane B, Stokstad ELR. Vitamin B12-folate interrelationships. *Annu Rev Nutr*. 1985;5(1):115-141. doi:10.1146/annurev.nu.05.070185.000555

215. Carter F, Heller P, Schafner G, Korn R. Formiminoglutamic acid (FIGLU) excretion in hepatic cirrhosis. *Arch Intern Med*. 1961;108(1):41-46. doi:10.1001/archinte.1961.03620070043006

216. Niederwieser A, Giliberti P, Matasović A, Pluznik S, Steinmann B, Baerlocher K. Folic acid non-dependent formiminoglutamic aciduria in two siblings. *Clin Chim Acta*. 1974;54(3):293-316. doi:10.1016/0009-8981(74)90248-4

217. Tan A, Awaiye K. Use of internal standards in LC-MS bioanalysis. In: Li W, Zhang J, Tse and FL, eds. *Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols, and Regulations*. John Wiley & Sons, Inc.; 2013:217-227. doi:10.1002/9781118671276.ch17

218. van de Merbel NC. Quantitative determination of endogenous compounds in biological samples using chromatographic techniques. *TrAC Trends Anal Chem*. 2008;27(10):924-933. doi:10.1016/j.trac.2008.09.002

219. Visconti G, de Figueiredo M, Salamin O, *et al*. Straightforward quantification of endogenous steroids with liquid chromatography-tandem mass spectrometry: Comparing calibration approaches. *J Chromatogr B*. 2023;1226:123778. doi:10.1016/j.jchromb.2023.123778

220. Piraud M, Ruet S, Boyer S, *et al*. Amino acid profiling for the diagnosis of inborn errors of metabolism. *Methods Mol Biol*. 2011;708:25-53. doi:10.1007/978-1-61737-985-7_2

221. National Center for Biotechnology Information. PubChem Compound Summary for CID 33032, Glutamic Acid. Updated April 12, 2025. Accessed January 18, 2025. https://pubchem.ncbi.nlm.nih.gov/compound/Glutamic-Acid

222. National Center for Biotechnology Information. PubChem Compound Summary for CID 439233, Formiminoglutamic Acid. Updated April 12, 2025. Accessed April 17, 2025. https://pubchem.ncbi.nlm.nih.gov/compound/Formiminoglutamic-Acid

223. U.S. Food & Drug Administration (FDA). Bioanalytical method validation guidance for industry. Updated May, 2018. Accessed April 17, 2025. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

224. European Medicines Agency. ICH Guideline M10 on bioanalytical method validation and study sample analysis. Published July 27, 2022. Accessed April 17, 2025. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf

225. Tamura Y, Ohta H, Kagawa Y, *et al*. Plasma amino acid profiles in dogs with inflammatory bowel disease. *J Vet Intern Med*. 2019;33(4):1602-1607. doi:10.1111/jvim.15525

226. Sakai K, Maeda S, Yonezawa T, Matsuki N. Decreased plasma amino acid concentrations in cats with chronic gastrointestinal diseases and their possible contribution in the inflammatory response. *Vet Immunol Immunopathol*. 2018;195:1-6. doi:10.1016/j.vetimm.2017.11.001

227. Heinze CR, Larsen JA, Kass PH, Fascetti AJ. Plasma amino acid and whole blood taurine concentrations in cats eating commercially prepared diets. *J Am Vet Med Assoc*. 2009;235(10):1193-1193. doi:10.2460/javma.235.10.1193

228. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J Chromatogr B*. 2007;852(1-2):22-34. doi:10.1016/j.jchromb.2006.12.030

229. Leela-arporn R, Ohta H, Tamura M, *et al*. Plasma-free amino acid profiles in dogs with hepatocellular carcinoma. *J Vet Intern Med*. 2019;33(4):1653-1659. doi:10.1111/jvim.15512

230. Xu Y, Gutierrez JP, Lu TS, *et al.* Quantification of the therapeutic peptide exenatide in human plasma. SCIEX Technical Notes. Published 2014. Accessed April 17, 2025. https://sciex.com/content/dam/SCIEX/pdf/tech-notes/all/mass-spectrometry-cms_074674.pdf

231. Lording PM, Bellamy JE. Trimethoprim and sulfadiazine: adverse effects of long-term administration in dogs. *J Am Anim Hosp Assoc*. 1978;14:410-417.

232. Siemens Healthineers. Atellica IM folate instructions for use. Published November, 2023. Accessed April 06 2025. https://content.doclib.siemens-healthineers.com/rest/v1/view?document-id=1026503

233. Siemens Healthineers. Immulite 2000 folic acid instructions for use. Published August 4, 2020. Accessed April 06, 2025. https://content.doclib.siemens-healthineers.com/rest/v1/view?document-id=736144

234. The jamovi project. jamovi (Version 2.6) [Computer Software]. Retrieved from https://www.jamovi.org

235. Friedrichs KR, Harr KE, Freeman KP, *et al*. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 2012;41(4):441-453. doi:10.1111/vcp.12006

236. Kahn S, Fein SA, Brodsky I. Effects of trimethoprim on folate metabolism in man. *Clin Pharmacol Ther*. 1968;9(5):550-560. doi:10.1002/cpt196895550