PRECLINICAL EVALUATION OF COMMON MARKERS AND IOHEXOL FOR THE DEVELOPMENT OF INTESTINAL PERMEABILITY TESTS IN DOGS — Studies on laboratory Beagle dogs and Sprague-Dawley rats

Rafael Frías Beneyto
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PRECLINICAL EVALUATION OF COMMON MARKERS AND IOHEXOL FOR THE DEVELOPMENT OF INTESTINAL PERMEABILITY TESTS IN DOGS

— Studies on laboratory Beagle dogs and Sprague-Dawley rats

Rafael Frías Beneyto

DOCTORAL DISSERTATION

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UNIVERSITY OF HELSINKI
2013
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Painosalama Oy – Turku, Finland 2013
To my family
## CONTENTS

### ABSTRACT

6

### ABBREVIATIONS

7

### LIST OF ORIGINAL PUBLICATIONS

8

1. INTRODUCTION

9

2. REVIEW OF THE LITERATURE

11

2.1 Intestinal epithelium

11

2.1.1 Role of the intestinal epithelial barrier

11

2.1.2 Uptake of molecules across the intestinal epithelium

11

2.2 Measurement of intestinal permeability

12

2.2.1 Permeability markers

14

2.2.2 Routes of intestinal permeation

15

2.2.3 Intestinal permeability in disease

17

2.3 Intestinal permeability tests in dogs

18

2.3.1 Overview of testing methods

19

2.3.2 The $^{51}$Cr-EDTA test

19

2.3.3 The dual sugar test

22

2.3.4 Contrast media for permeability testing

25

3. AIMS OF THE STUDY

26

4. MATERIALS AND METHODS

27

4.1 Experimental animals

27

4.1.1 Dogs

27

4.1.2 Rats

28

4.2 Permeability testing in dogs

29

4.3 Permeability testing in rats

30

4.4 Measurement of permeability markers

31

4.5 Formulas to calculate the recovery rates of $^{51}$Cr-EDTA and iohexol

31

4.6 Statistical analysis (I–V)

32

5. RESULTS

34

5.1 Urinary measurements and statistical comparisons of the markers after their concurrent oral administration to healthy adult male Beagles (I)

34

5.2 Comparison between the $^{51}$Cr-EDTA test measured in blood and urine in healthy adult Beagles (II)

37

5.3 Comparison between the $^{51}$Cr-EDTA blood test measured in serum and plasma in healthy adult Beagles (III)

38

5.4 Comparison between $^{51}$Cr-EDTA and iohexol as permeability blood markers in healthy adult Beagles (IV)

39
5.5 Evaluation of iohexol as a permeability marker in healthy rats and in rats with DSS-induced inflammatory bowel disease (V) ...........................................41

6. DISCUSSION ........................................................................................................43
   6.1 Reference ranges and comparisons between the most relevant permeability markers in healthy adult Beagles (I) .................................................................43
   6.2 $^{51}$Cr-EDTA versus lactulose and sugar probes for intestinal permeability measurements (I) .................................................................................................44
   6.3 A single marker versus a combination of two for intestinal permeability measurements (I) ........................................................................................................45
   6.4 The $^{51}$Cr-EDTA blood test in Beagles (II, III) ....................................................46
   6.5 Iohexol as a potential intestinal permeability marker in dogs (IV) .......................47
   6.6 Preclinical evaluation of iohexol as intestinal permeability marker using a well-characterized experimental intestinal disease rat model (V) .................47

7. CONCLUSIONS ....................................................................................................49

8. ACKNOWLEDGEMENTS ....................................................................................51

REFERENCES ............................................................................................................53

ORIGINAL PUBLICATIONS ....................................................................................59
Abstract

Preclinical evaluation of common markers and iohexol for the development of intestinal permeability tests in dogs. — Studies on laboratory Beagle dogs and Sprague-Dawley rats. Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Finland.

Intestinal permeability testing is the specific method to assess for a defective intestinal epithelial barrier. Intestinal permeability measurements are considered helpful and non-invasive means to evaluate intestinal mucosal damage for both scientific (particularly) and clinical purposes, and have been widely used in laboratory rodents and humans. Despite their many advantages, permeability tests have not gained widespread use as a testing option for the detection and management of canine intestinal disorders in veterinary clinical research. The main reasons for this may include the lack of an optimal biomarker for permeability testing, impracticalities involving current testing methodologies, and inconsistencies in test results that have been found by investigators using these tests.

Chromium 51-labeled ethylenediamine tetra-acetic acid (\(^{51}\)Cr-EDTA) is widely considered the most accurate intestinal permeability probe, but the use of radioactivity is a major drawback. Sugar biomarkers such as lactulose and rhamnose have been more commonly used in the recent years, but they have been associated with marked inconsistencies in the test results. Iohexol is a contrast medium commonly used in radiology for diagnostic purposes in human and veterinary patients, but this molecule has more recently been successfully used for the screening of gut mucosal damage in laboratory rats and humans. The main advantage of iohexol is that its use does not involve radioactivity, nor is it degraded in the intestinal lumen. Furthermore, it has the potential to be quantified by different analytical techniques.

The main objective of this project was to improve the methodology of the intestinal permeability tests in dogs in order to make the testing simpler, more practical and accurate for veterinarians and researchers using this approach to investigate intestinal mucosal damage and disorders associated with a defective intestinal epithelial barrier. An additional objective was to preliminarily assess the use of iohexol as a novel intestinal permeability marker for use in dogs. The work consisted of preclinical comparisons of the most relevant intestinal permeability markers including \(^{51}\)Cr-EDTA, lactulose, and rhamnose, and iohexol performed in both urine and blood tests using laboratory dog and rat models.

In conclusion, studies on the percentage urinary recovery of \(^{51}\)Cr-EDTA, lactulose, and rhamnose, as well as D-xylose, 3-O-methyl-D-glucose, and sucrose after their oral simultaneous administration provided normative data for healthy adult male Beagle dogs. The analysis revealed a discrepancy in the percentage urinary recovery between \(^{51}\)Cr-EDTA and lactulose, suggesting that these two markers are not as equivalent as has so far been believed based on previous studies in humans and cats. It was also concluded that the use of a single marker provides comparable test results to the use of two markers, as evidenced by a comparison of recovery values from \(^{51}\)Cr-EDTA and lactulose versus their correspondent ratio against rhamnose. This supports the hypothesis that, in contrast to the dual sugar test, the use of one inert larger probe may be sufficient for permeability testing, and the testing procedure may consequently be considerably simplified. The studies also demonstrated that the \(^{51}\)Cr-EDTA permeability blood test based on the collection of at least two serum or plasma specimens gives comparable results to the 6-h cumulative urine test. The blood approach is much easier than the urine-based test, as it avoids the constraints associated with urine collection in dogs. Iohexol was shown to have a clear relationship with \(^{51}\)Cr-EDTA in serum levels when they were simultaneously administered to Beagle dogs. When it was used as an intestinal permeability probe in laboratory rats before and after the induction of a well-characterized experimental form of inflammatory bowel disease, it was also possible to clearly discriminate between healthy animals and rats with intestinal mucosal damage. The iohexol blood test can therefore be considered a promising tool for assessing canine intestinal permeability in veterinary clinical research. Nevertheless, further studies using iohexol as intestinal permeability blood marker, particularly in diseased dogs, are warranted before firm conclusions can be made on the validity of this test.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}\text{Cr-EDTA}$</td>
<td>chromium-labeled ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>$^{99m}\text{Tc-DTPA}$</td>
<td>$^{99m}\text{Tc}$-diethylenetriaminopentaacetate</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sulfate sodium</td>
</tr>
<tr>
<td>EPI</td>
<td>Exocrine pancreatic insufficiency</td>
</tr>
<tr>
<td>G</td>
<td>3-O-methyl-D-glucose</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>H</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HPLC-PAD</td>
<td>High-performance liquid chromatography-pulsed amperometric detection</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IP</td>
<td>Intestinal permeability</td>
</tr>
<tr>
<td>L</td>
<td>Lactulose</td>
</tr>
<tr>
<td>L/R</td>
<td>Lactulose-to-rhamnose ratio</td>
</tr>
<tr>
<td>MBq</td>
<td>Megabecquerel</td>
</tr>
<tr>
<td>MM</td>
<td>Molecular mass</td>
</tr>
<tr>
<td>mSv</td>
<td>Milli-Sieverts</td>
</tr>
<tr>
<td>µCi</td>
<td>Microcurie</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed amperometric detection</td>
</tr>
<tr>
<td>PO</td>
<td>Per os</td>
</tr>
<tr>
<td>R</td>
<td>Rhamnose</td>
</tr>
<tr>
<td>S</td>
<td>Sucrose</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the median</td>
</tr>
<tr>
<td>SIBO</td>
<td>Small intestinal bacterial overgrowth</td>
</tr>
<tr>
<td>TJs</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TBV</td>
<td>Total blood volume</td>
</tr>
<tr>
<td>TPV</td>
<td>Total plasma volume</td>
</tr>
<tr>
<td>TSV</td>
<td>Total serum volume</td>
</tr>
<tr>
<td>TTSV</td>
<td>Total test solution volume</td>
</tr>
<tr>
<td>TUV</td>
<td>Total urine volume</td>
</tr>
<tr>
<td>X</td>
<td>D-xylose</td>
</tr>
<tr>
<td>X/G</td>
<td>D-xylose/3-O-methyl-D-glucose ratio</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS


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1. INTRODUCTION

The breakdown of intestinal mucosal integrity is a pathological condition that is frequently accompanied by damage to the intercellular tight junctions and ultimately leads to increased intestinal permeability. Hyperpermeability is believed to play a key role in initiating and developing intestinal and non-intestinal diseases such as inflammatory bowel disease, irritable bowel syndrome, type I diabetes, food allergies, and several other disorders in humans and animals, including dogs. (Hall, 1999, Farhadi et al., 2003, Clayburgh et al., 2004, Teshima and Meddings, 2008, Fasano, 2012a, Suzuki, 2012, Teshima et al., 2012)

In dogs, the common method for assessing the status of intestinal mucosal integrity has been histopathological examination of tissue samples that are collected from the gut, but this approach is often not the most appropriate or sensitive for various reasons, including diagnostic and practical drawbacks. Histopathology as diagnostic tool for intestinal permeability may be flawed, because it is known that derangement of the canine bowel mucosa often results in increased intestinal permeability while not causing histopathological abnormalities, even in individuals with severe clinical symptoms. (Hall and Batt, 1990, Bjarnason et al., 1995, Batt, 2000, Willard et al., 2002, Willard and Mansell, 2011) In addition, histopathology also requires an invasive approach for sampling and collecting tissue samples from the relatively inaccessible canine gut, and the use of invasive methods and anesthesia is always required. This invasiveness reduces animal welfare and may be especially inappropriate in long-term clinical follow-ups and in longitudinal research studies, where serial biopsies are taken in the same individual over the study time.

Intestinal permeability testing, on the other hand, has proved to be valuable in the diagnosis and follow-up of a variety of clinical diseases and experimental conditions for which intestinal mucosal integrity must be evaluated. Intestinal permeability tests have shown many advantages, including ease of performance (consists of the oral administration of a marker and the subsequent recovery of a sample in urine or blood), a low level of invasiveness, and objective assessment of mucosal integrity (i.e. provides a numerical index of mucosal pathology, which enables a qualitative and quantitative measure of gut damage). Additionally, permeability tests can help in reducing and refining the use of dogs in research. This type of testing may be possible to repeat in the same dog without significantly jeopardizing its welfare and thereby avoiding the need to use additional dogs in the same study, and is also able to replace more invasive techniques such as endoscopy or laparotomy, which are needed to collect a tissue biopsy for subsequent histopathological analysis.

However, despite the apparent value of intestinal permeability tests in revealing and examining intestinal mucosal damage, these tests are not frequently used by veterinarians
or biomedical scientists working with dogs. This is most likely due to a number of flaws recognized in the permeability testing methodology, such as relevant downsides of the molecules generally used as biomarkers and certain impracticalities of the test.

Permeability testing in dogs was initially performed using chromium 51-labeled ethylenediamine tetra-acetic acid ($^{51}$Cr-EDTA) as a probe. This marker is still considered the gold standard molecule for intestinal permeability measurements, but the use of and exposure to radioactivity is a considerable limiting factor that has precluded this testing option, especially in the clinical setting. To avoid problems associated with radioactive labels, intestinal permeability testing using sugar markers such as lactulose and rhamnose has been more commonly applied in the more recent years. However, measurements using saccharides have been associated with conflicting test results, and the use of this testing option in dogs is currently also declining. (Hall, 1999, Suchodolski and Steiner, 2003, Batt, 2009, Berghoff, 2011) More recently, iohexol, a radiocontrast medium commonly used in medical imaging, has been successfully used as an intestinal permeability marker for non-invasive screening of intestinal damage in laboratory rats and humans. Iohexol meets the criteria described for permeability markers, but in contrast to $^{51}$Cr-EDTA and the combination of sugars, this molecule does not involve radioactivity, nor is inconsistently degraded by intestinal bacteria. Furthermore, it has the added advantages of being inexpensive compared to the other molecules, widely available in radiology departments, and of being potentially quantified by different analytical techniques. (Stordahl, 1988a, b, Andersen et al., 1992, Halme et al., 1993, Andersen and Laerum, 1995, Halme et al., 1997, Halme et al., 2000, Andersen et al., 2001, Kishimoto et al., 2010)

The main objective of the research described in this thesis was to perform a basic comparative study on previously used and potential markers of intestinal permeability in dogs. The ultimate goal was to make this testing more easily accessible and reliable to clinicians and scientists investigating important canine intestinal and extra-intestinal diseases and conditions.
2. REVIEW OF THE LITERATURE

2.1 Intestinal epithelium

2.1.1 Role of the intestinal epithelial barrier

The luminal surface of the gastrointestinal tract is covered by a single layer of polarized and differentiated epithelial cells tightly connected by intercellular junctions. The main function of this epithelial layer is to form a dynamic barrier that enables the absorption of nutrients, water, ions, vitamins and electrolytes from the external environment into the mammalian host. Most of this uptake occurs in the small intestine. Another function of the intestinal mucosal barrier is to prevent the uncontrolled entrance of potentially damaging compounds such as foreign antigens, carcinogens, pathogens, and toxins from the lumen into the submucosa and the blood circulation (Figure 1). (Kararli, 1995, Daugherty and Mrsny, 1999, Groschwitz and Hogan, 2009, Catalioto et al., 2011)

![Figure 1](image_url)

**Figure 1.** Schematic diagram of the dual function of the intestinal epithelium: A) uptake of beneficial molecules; and B) a barrier against harmful materials.

2.1.2 Uptake of molecules across the intestinal epithelium

Figure 2 schematically illustrates the uptake of molecules across the intestinal epithelium. The passage of luminal substances across the intestinal epithelium essentially takes place by absorption or permeation. Absorption is based on the transportation of molecules
across the epithelial plasma membrane using an active or facilitated mechanism by specific carrier proteins or endocytosis, and this process takes place via a transcellular route. Permeation is based on the non-mediated movement of solutes through the intestinal epithelium by means of passive diffusion, predominantly following paracellular channels but also using smaller transcellular pores.

The paracellular route is considered the most important means for the permeation of small hydrophilic molecules, and its regulation is determined by intercellular junctions that bridge the apicolateral border of the epithelial cells. The role of the apical tight junctions in the paracellular route is critical in limiting access of harmful substances to host tissues and the systemic circulation by reducing the space between adjacent cells and the passage of charge entities. (Clayburgh et al., 2004, Groschwitz and Hogan, 2009, Ménard et al., 2010, Catalioto et al., 2011, Shen et al., 2011, Fasano, 2012a)

The ability to selectively (rather than absolutely) restrict the non-mediated passive diffusion of materials through the paracellular pathway is referred to as intestinal permeability. (Travis and Menzies, 1992) This concept mainly refers to the passage of ions and small hydrophilic inert molecules of low molecular weight.

**Figure 2.** Uptake of molecules across the intestinal epithelium.

### 2.2 Measurement of intestinal permeability

Figure 3 schematically illustrates how intestinal permeability can be measured *in vivo*. Traditionally, measurement of intestinal permeability is performed by orally administering specific permeability markers, such as $^{51}$Cr-EDTA, or lactulose and...
rhamnose. The concentration of the recovered markers is then determined, usually in urine, after a timed period of 6 to 24 hours, or more rarely in blood after 2 hours. (Hall et al., 1989, Bjarnason et al., 1995, Sørensen et al., 1997, Hall, 1999, Suchodolski and Steiner, 2003, Arrieta et al., 2006, Batt, 2009, Rodriguez et al., 2009b)

**Figure 3.** Schematic diagram of the principle of gastrointestinal permeability testing. After the oral administration of permeability markers, the molecules will cross the epithelium, reach the blood system, and then will be eliminated in urine. The markers are quantified in urine after 6 to 24 hours, or in blood after 2 hours. Increased permeability is reflected by an increased recovery of the marker.

Under physiological circumstances, the permeability markers pass across the intestinal mucosa in a restricted manner. In conditions where the mucosal lining is damaged or lost, the passage of permeability probes across the gut barrier occurs freely. (Hall et al., 1989, Bjarnason et al., 1995, Bradford et al., 2012) The urinary excretion or blood concentrations of the orally-administered markers are influenced by their metabolism within the organism and by the glomerular filtration rate. Hence, the biomarkers should be completely inert and the test should not be performed in dogs with high serum creatinine or urea concentrations. (Hall et al., 1989, Hall, 1999, Suchodolski and Steiner, 2003, Batt, 2009)

The **urine test** for intestinal permeability testing in dogs necessitates placement of the subject in a metabolism cage to ensure complete urine collection and to prevent contamination of the urine with feces containing remains of the non-absorbed biomarker. Contamination of urine by feces may occur in the urine-based test, especially in dogs with diarrhea, a typical disorder in patients with intestinal mucosal damage. If fecal contamination occurs, the results of the testing are considered invalid and the test must be cancelled and repeated again after a period of at least 5 days. In dogs, this test method was initially performed using a cumulative 24-h urinary excretion of the permeability marker. Later, a cumulative 6-hour excretion of the marker was demonstrated to be as sensitive as a 24-h collection period. Collecting the urine for 6 hours is more advantageous, because a shorter collection period is less stressful for the dog, helps
reducing the probability of fecal contamination of urine, and more specifically reflects small intestinal permeability. (Hall et al., 1989, Marks and Williams, 1998, Hall, 1999)

A blood-based permeability test using sugars as probes has also been developed for use in canine patients. This approach obviates the problems associated with urine collection and reduces test time from six to two hours. However, this approach has not gained wide popularity due to the analytical constraints of the sugars in plasma samples. (Sørensen et al., 1993, Sørensen et al., 1997, Hall, 1999)

2.2.1 Permeability markers

Optimal intestinal permeability probes should have specific desirable physicochemical features, including those listed in Table 1. The probe molecules follow first-order kinetics of permeation and should be water-soluble, small, non-toxic, non-degradable, and not metabolized before, during, or after permeating the intestine. Additionally, they should not be naturally present in body fluids. The urinary excretion of probes should be complete following intravenous injection, and their quantification should be sensitive, accurate, and easy. (Chadwick et al., 1977, Ukabam et al., 1983, Bjarnason et al., 1995, Andersen et al., 2001)

Table 1. Optimal features of intestinal permeability probes. Modified from Andersen et al., 2001.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport across intestinal epithelium by permeation</td>
<td>Hydrophilic, lipophobic</td>
</tr>
<tr>
<td>Small (specific cross-sectional diameter and molecular weight)</td>
<td>Non-charged</td>
</tr>
<tr>
<td>Non-metabolizable by internal processes</td>
<td>Resistant to intestinal conditions (i.e. non-degradable)</td>
</tr>
<tr>
<td>Safe (i.e. non-toxic, innocuous)</td>
<td>Not recognized by the immune system</td>
</tr>
<tr>
<td>Artificial (i.e. not present in normal body fluids)</td>
<td>Fully (and rapidly) excreted in urine after intravenous injection</td>
</tr>
<tr>
<td>Accurately and easily quantified</td>
<td>Inexpensive</td>
</tr>
<tr>
<td>Widely available</td>
<td></td>
</tr>
</tbody>
</table>

Various molecules have been used as permeability probes (Table 2), but the majority of work in humans, dogs, and experimental animals has employed non-degraded radiolabeled chelates ($^{51}$Cr-EDTA) and small sugar probes expressed as a ratio such as disaccharides (lactulose, cellobiose) and monosaccharides (rhamnose, mannitol). Although their use is generally not recommended, in the past, polyethylene glycols (PEG 400) of different molecular masses were also employed (Maxton et al., 1986, Bjarnason et al., 1995). More recently, radiocontrast media (iohexol, ioxidanol) have additionally been successfully utilized as permeability markers in humans and laboratory rats.
Table 2. Cross-sectional diameter and weight of selected intestinal permeability markers.

<table>
<thead>
<tr>
<th>Class</th>
<th>Probe</th>
<th>Molecular size Å (nm)</th>
<th>Molecular mass Da (g/mol)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiolabeled chemicals</td>
<td>$^{51}$Cr-EDTA</td>
<td>10.5 (1.05)</td>
<td>341</td>
<td>Hollander et al., 1988</td>
</tr>
<tr>
<td>Disaccharides</td>
<td>Lactulose</td>
<td>9.5 (0.95)</td>
<td>342</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellobiose</td>
<td>10.3 (1.03)</td>
<td>342</td>
<td></td>
</tr>
<tr>
<td>Monosaccharides</td>
<td>Rhamnose</td>
<td>8.3 (0.83)</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>6.7 (0.67)</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>Contrast media</td>
<td>Iohexol</td>
<td>12 (1.2)</td>
<td>821</td>
<td>Andersen et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Iodixanol</td>
<td>15 (1.5)</td>
<td>1550</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Routes of intestinal permeation

In brief, permeation across the intestinal epithelium takes place through the paracellular space between adjacent enterocytes and through the cell membranes via a transcellular route. The route that a molecule uses for permeation generally depends on its shape, size, weight, and charge. It is considered that small hydrophilic solutes permeate across the paracellular space, whereas hydrophobic compounds traverse the epithelium mainly via a transcellular pathway. (Bjarnason et al., 1995, He et al., 1998, Linnankoski et al., 2010, Shen et al., 2011)

The most straightforward hypothesis regarding the routes and rates of permeability markers crossing the gut epithelium has been provided by Fihn and co-workers (Fihn et al., 2000). Their data suggest that there are three spatially separated aqueous pores of different dimensions and density distributed along the crypt–villus axis. The tips of villi contain abundant small pores (radius < 6 Å; < 0.6 nm), the bases of the villi have fewer intermediate-sized pores (10-15 Å ; 1–1.5 nm), and the crypts have sparse large pores (50-60 Å; 5–6 nm) (Figure 4). The aqueous pores at the villous tip are susceptible to solvent drag effects (recirculation of water between the crypts and the villus), whereas the channels at the base of the villi and in the crypts are not affected. This hypothesis is consistent with data from other investigators evidencing that the tight junctions of adjacent enterocytes are tighter at the villous tips than in the crypts. Similarly, the junctions become progressively tighter from the small to the large intestinal tract. (Madara et al., 1980, Maxton et al., 1986, Hollander, 1992, Travis and Menzies, 1992, Bjarnason et al., 1995, Rouge et al., 1996, Fihn et al., 2000, Van Itallie et al., 2008, Linnankoski et al., 2010, Shen et al., 2011).

In dogs, the exact size of the intercellular space is unknown, but there is evidence suggesting that dogs have a wider extracellular space than humans or rats. (Hall and Batt, 1990, He et al., 1998)
It has now become accepted that small permeability markers the size of mannitol are able to permeate with relative freedom throughout the villus-crypt axis, whereas the permeation of larger markers such as $^{51}$Cr-EDTA or lactulose occurs at the base of the villus and in the crypts, where the tight junctions are leakier and the intercellular channels are wider. (Maxton et al., 1986, Hall et al., 1989, Hall and Batt, 1990, Bjarnason et al., 1995, Arrieta et al., 2006, Ménard et al., 2010)

At present, it is also now clearer that paracellular permeability channels are not static and they are largely influenced by the regulation of intercellular junctions. The most important of these complex intercellular junctions are the apical tight junctions, but adherens junctions and desmosomes also play an important role (Figure 5). The apical tight junctions are considered the principal determinants of cell–cell proximity and thus intestinal permeability. They consist of a complex cluster of numerous transmembrane proteins, including claudins, occludin, and junctional adhesion molecule A, being connected to the actomyosin ring through zonula occludens proteins. All these structures form a network that connects neighboring enterocytes, and are known to be highly dynamic. They are regulated in response to numerous extracellular stimuli such as

---

**Figure 4.** Schematic diagram of the number and size of intercellular pores across the crypt–villus axis.

---

**PORE SIZE**

- SMALL (< 9.5 Å)
- MEDIUM (10-20 Å)
- LARGE (50-60 Å)

**PORE NUMBERS**

- HIGH DENSITY
- INTERMEDIATE DENSITY
- LOW DENSITY
Review of the Literature

17


Figure 5. Schematic diagram of the paracellular route and intercellular junctions.

2.2.3 Intestinal permeability in disease

The loss of epithelial layer cells as a consequence of apoptosis, or intestinal mucosal damage leads to the disruption of epithelial barrier integrity, which is reflected by an overall increased intestinal permeability. An elevated permeability secondary to mucosal injury is primarily characterized by alterations in the tight junction integrity and function, leading to an increased leakiness of the paracellular space. The issue of whether hyperpermeability is a cause or consequence of certain disease states is still a matter of debate, but currently it is well accepted that a high intestinal permeability has pathophysiological significance in the development of various intestinal (e.g. inflammatory bowel disease) and systemic (e.g. autoimmune) diseases. This because enhanced intestinal permeability can result in mucosal penetration of damaging compounds such as antigens, proteases, hydrogen ions, and bacteria and their products from the gut lumen to the subepithelial lamina propria. This may elicit a variety of pathological processes, such as direct toxicity to the structure and function of the mucosa, leading, for example, to a loss of protein from the vessels into the intestinal lumen, causing hypoproteinemia. More importantly, it may lead to the stimulation of the mucosal immune system and infiltration of inflammatory cells, which will amplify and perpetuate the host defense response, leading to chronicity of

2.3 Intestinal permeability tests in dogs

Tests of intestinal permeability in dogs have been applied to various clinical research conditions and proved to be useful and sensitive in the detection of intestinal mucosal damage, in confirming diagnoses, predicting the prognosis of certain enteropathies, monitoring the response to treatment of intestinal disease, evaluating the effect of possible enterotoxins (e.g. non-steroidal anti-inflammatory drugs), and in quantifying inherent differences in intestinal permeation between different species and dog breeds. Table 3 provides examples of experimental and clinical conditions in which an altered intestinal permeability has been demonstrated in dogs.

Table 3. Experimental and clinical conditions reported to be associated with increased gastrointestinal permeability in dogs.

<table>
<thead>
<tr>
<th>Condition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten-sensitive enteropathy</td>
<td>Hall and Batt, 1990, Hall and Batt, 1991a, Hall and Batt, 1991b,</td>
</tr>
<tr>
<td></td>
<td>Hall and Batt, 1991c, Garden et al., 1997, Garden et al., 1998,</td>
</tr>
<tr>
<td></td>
<td>Manners et al., 1994, Garden et al., 1995</td>
</tr>
<tr>
<td>Small intestinal bacterial overgrowth</td>
<td>Hall and Batt, 1990, Morris et al., 1994, Rutgers et al., 1996</td>
</tr>
<tr>
<td>Intestinal parasitism (e.g. Giardia spp.)</td>
<td>Hall and Batt, 1990</td>
</tr>
<tr>
<td>Dietary hypersensitivity and intolerance</td>
<td>Rutgers et al., 1995</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Sørensen et al., 1997, Kobayashi et al., 2007</td>
</tr>
<tr>
<td>Species and breed differences</td>
<td>Randell et al., 2001</td>
</tr>
<tr>
<td>Juvenile and adult age</td>
<td>Weber et al., 2002</td>
</tr>
<tr>
<td>Small and large body size</td>
<td>Weber et al., 2002</td>
</tr>
<tr>
<td>Traumatic injury</td>
<td>Streeter et al., 2002</td>
</tr>
<tr>
<td>Intestinal viruses (e.g. parvovirus)</td>
<td>Mohr et al., 2003</td>
</tr>
<tr>
<td>Strenuous exercise</td>
<td>Davis et al., 2005</td>
</tr>
<tr>
<td>Gut-injury assessment of nonsteroidal anti-inflammatory drugs (aspirin and meloxicam)</td>
<td>Meddings et al., 1995, Roskar et al., 2011</td>
</tr>
</tbody>
</table>

Intestinal permeability and function testing in dogs has failed to become routinely used in small animal practice, and has been inconsistently used for research purposes. This is probably due to problems associated with impracticalities in the testing
protocols employed, and also because of conflicting test results observed in certain studies that could not be explained on the basis of different analytical techniques or testing protocols. Although intestinal permeability tests are generally considered sensitive in detecting intestinal mucosal damage, permeability measurements have not been considered specific for a definitive diagnosis, because permeability may be increased in primary intestinal diseases as well as in many non-intestinal disorders. (Hall, 1999, Suchodolski and Steiner, 2003, Allenspach et al., 2006, Batt, 2009, Berghoff, 2011)

2.3.1 Overview of testing methods

Table 4 presents studies in which intestinal permeability tests have been performed in dogs and summarizes the most relevant findings of such studies. Permeability testing in dogs was first applied using $^{51}$Cr-EDTA as a single probe. This marker is still considered the reference molecule for intestinal permeability measurements, but exposure to radioactivity is a limiting factor that has considerably affected its application. Permeability testing using two different-sized sugar probes such as lactulose and rhamnose has become the method most frequently used by clinical investigators for assessing small intestinal permeability in dogs. The ratio of two sugars does not involve the use of radioactivity, and has been generally thought to provide a more reliable permeability index than using $^{51}$Cr-EDTA alone. However, findings from more recent permeability investigations using saccharides as probe markers have provided doubtful test results, and as a consequence, their use in dogs is currently also declining. (Hall, 1999, Suchodolski and Steiner, 2003, Allenspach et al., 2006, Batt, 2009, Berghoff, 2011)

2.3.2 The $^{51}$Cr-EDTA test

$^{51}$Cr-EDTA is a radiolabeled chelate with a half-life of approximately 28 days that fulfills most of the theoretical requirements for an ideal intestinal permeability probe. Intestinal permeability testing using this molecule as a single marker has been considered the gold standard method for permeability measurements in clinical research studies.

$^{51}$Cr-EDTA is water-soluble, metabolically inert (not affected by bacterial activity and resistant to intestinal hydrolization), confined to extracellular fluid, completely and rapidly excreted by the kidneys, straightforward to assay, extremely stable, relatively safe (weak gamma emitter, does not bind to biological materials, lacks chemical toxicity, and the estimated radiation dose for the human patient is minimal), and providing care is taken in performing this test in dogs, the hazard to personnel is minimal. (Chantler et al., 1969, Løkkken, 1970, Ahrens and Aronson, 1971, Bjarnason et al., 1983a, Bjarnason et al., 1983b, c, Elia et al., 1987, Hall et al., 1989, Katz and Hollander, 1989, Hall and Batt, 1990, Lifschitz and Shulman, 1990, Bjarnason et al., 1995)
Table 4. Intestinal permeability tests used in dogs.

<table>
<thead>
<tr>
<th>Method</th>
<th>Study</th>
<th>Most relevant findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>^{51}Cr-EDTA</td>
<td>Hall et al., 1989</td>
<td>• Test validation on a heterogeneous group of clinically healthy adult dogs.</td>
</tr>
<tr>
<td></td>
<td>Hall and Batt, 1990</td>
<td>• Increased permeability associated with small intestinal disease (gluten-sensitive enteropathy, giardiasis and SIBO) in a heterogeneous group of dogs.</td>
</tr>
<tr>
<td></td>
<td>Hall and Batt, 1991a</td>
<td>• Primary permeability defect in the pathogenesis of gluten-sensitive enteropathy in Irish Setters.</td>
</tr>
<tr>
<td></td>
<td>Batt et al., 1992</td>
<td>• Enhanced permeability as a consequence of SIBO in apparently healthy Beagles.</td>
</tr>
<tr>
<td></td>
<td>Marks and Williams, 1998</td>
<td>• Six-hour urinary recovery as an alternative to 24-h recovery.</td>
</tr>
<tr>
<td></td>
<td>Vaden et al., 2000</td>
<td>• Permeability was not increased in six Soft-Coated Wheaten Terriers of both sexes with familial protein-losing enteropathy, protein-losing nephropathy, or both, when test results were compared with four random-source male dogs used as controls.</td>
</tr>
<tr>
<td>Cellobiose and mannitol</td>
<td>Hall and Batt, 1991c</td>
<td>• Use on Irish Setters with gluten-sensitive enteropathy.</td>
</tr>
<tr>
<td></td>
<td>Vaden et al., 2000</td>
<td>• Permeability was not increased in six Soft-Coated Wheaten Terriers of both sexes with familial protein-losing enteropathy, protein-losing nephropathy, or both, when test results were compared with four random-source male dogs used as controls.</td>
</tr>
<tr>
<td>Lactulose and rhamnose</td>
<td>Rutgers et al., 1992</td>
<td>• Use on dogs with diet-sensitive intestinal disease.</td>
</tr>
<tr>
<td></td>
<td>Elwood et al., 1993</td>
<td>• A multiple sugar combination for intestinal permeability and function testing in dogs.</td>
</tr>
<tr>
<td></td>
<td>Quigg et al., 1993</td>
<td>• Increased permeability in heterogeneous group of dogs with gastrointestinal disease with panhypoproteinemia.</td>
</tr>
<tr>
<td></td>
<td>Sørensen et al., 1993</td>
<td>• Development of high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) for the quantification of lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in the urine of a heterogeneous group of healthy dogs.</td>
</tr>
<tr>
<td></td>
<td>Morris et al., 1994</td>
<td>• Increased permeability in laboratory Beagles with repeated episodes of diarrhea associated with SIBO after experimental renal surgery and dietary change.</td>
</tr>
<tr>
<td></td>
<td>Rutgers et al., 1995</td>
<td>• Abnormal permeability in dogs with dietary hypersensitivity and intolerance.</td>
</tr>
<tr>
<td></td>
<td>Rutgers et al., 1996</td>
<td>• Successful treatment was matched with significant reductions in permeability.</td>
</tr>
<tr>
<td></td>
<td>Sørensen et al., 1997</td>
<td>• Development of HPLC-PAD for the quantification of lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in the plasma of a heterogeneous group of healthy dogs, dogs with IBD, and dogs with SIBO.</td>
</tr>
<tr>
<td>Method</td>
<td>Study</td>
<td>Most relevant findings</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Lactulose and</td>
<td>Garden et al., 1997</td>
<td>- Reference range and repeatability for urine test in healthy adult Irish Setters.</td>
</tr>
<tr>
<td>rhamnose</td>
<td></td>
<td>- No significant differences in permeability according to sex or age in adults.</td>
</tr>
<tr>
<td></td>
<td>Garden et al., 1998</td>
<td>- Intrinsic differences were shown in permeability between Irish setters and a heterogeneous group of control dogs of other breeds maintained in similar environmental conditions.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Both urine and blood tests were used, but rhamnose recovery did not correlate between the two methods.</td>
</tr>
<tr>
<td></td>
<td>Steiner et al., 2000</td>
<td>- Development of anion-exchange HPLC-PAD for the quantification of sucrose, lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in the urine of dogs.</td>
</tr>
<tr>
<td></td>
<td>Randell et al., 2001</td>
<td>- Permeability differs between healthy dogs of different breeds, and between dogs and cats.</td>
</tr>
<tr>
<td></td>
<td>Steiner et al., 2002</td>
<td>- Kinetic study of sucrose, lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in healthy male Beagles, showing that a 6-h urine collection period is sufficient for gastrointestinal permeability testing.</td>
</tr>
<tr>
<td></td>
<td>Streeter et al., 2002</td>
<td>- Permeability is altered in dogs with traumatic injury.</td>
</tr>
<tr>
<td></td>
<td>Weber et al., 2002</td>
<td>- Puppies (12 weeks old) have higher permeability than adults (60 weeks old).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Large-sized dogs had higher permeability than small dogs.</td>
</tr>
<tr>
<td></td>
<td>Mohr et al., 2003</td>
<td>- Increased permeability in dogs with acute parvoviral infection.</td>
</tr>
<tr>
<td></td>
<td>Davis et al., 2005</td>
<td>- Increased permeability in racing Alaskan sled dogs after sustained strenuous exercise, which returned to normal within 2 weeks of normal activity.</td>
</tr>
<tr>
<td></td>
<td>Royer et al., 2005</td>
<td>- Increased permeability in stressed racing Alaskan dogs with gastric ulcers.</td>
</tr>
<tr>
<td></td>
<td>Allenspach et al., 2006</td>
<td>- No significant differences in permeability in a heterogeneous group of dogs with chronic enteropathies before and after treatment.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Permeability tests did not correlate with histological findings.</td>
</tr>
<tr>
<td></td>
<td>Kobayashi et al., 2007</td>
<td>- Increased permeability in a heterogeneous group of dogs with lymphocytic-plasmacytic enteritis compared to healthy control dogs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Permeability tests correlated with histological findings.</td>
</tr>
<tr>
<td></td>
<td>Craven et al., 2007</td>
<td>- Absence of increased permeability in a heterogeneous group of client-owned dogs before, during, and after receiving meloxicam or carprofen.</td>
</tr>
<tr>
<td></td>
<td>Rodriguez et al., 2009</td>
<td>- Kinetic study of sucrose, lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in dog serum after orogastric administration.</td>
</tr>
<tr>
<td></td>
<td>Rodriguez et al., 2009b</td>
<td>- Development of gas chromatography-mass spectrometry for the quantification of sucrose, lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in canine serum.</td>
</tr>
<tr>
<td>Lactulose and</td>
<td>Roskar et al., 2011</td>
<td>- Increased gastrointestinal permeability in Beagles during and after receiving meloxicam was demonstrated using a plasma-based test.</td>
</tr>
<tr>
<td>mannitol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The $^{51}\text{Cr-EDTA}$ test was validated as a urine-based test for canine use in 1989 (Hall et al., 1989), and has subsequently proved valuable in the detection of mucosal damage in dogs affected with gluten-sensitive enteropathy, small intestinal bacterial overgrowth, and giardiasis (Hall and Batt, 1990). The urine test needs the placement of the subject in a metabolism cage for 6 to 24 hours to ensure complete urine collection, and to prevent fecal contamination of the urine. Clinically healthy dogs excreted less than 17% of the orally administered dose of $^{51}\text{Cr-EDTA}$ in a 24-h urinary recovery test (Hall et al., 1989), whereas less than 12% was excreted in a 6-h urinary recovery test (Marks and Williams, 1998).

The $^{51}\text{Cr-EDTA}$ test is typically carried out by using a single marker only. This has been thought to affect the test’s sensitivity, as non-mucosal factors such as renal function, intraluminal dilution and intestinal transit time could all affect, at least in theory, the urinary recovery of the orally administered probe. Nevertheless, the optimal physicochemical characteristics of $^{51}\text{Cr-EDTA}$ are believed to compensate this inconvenience when using it in permeability studies. (Bjarnason et al., 1995, Hall, 1999) However, the main drawbacks of using $^{51}\text{Cr-EDTA}$ and the main cause of its current unpopularity are associated with the costs of the radioisotope and the equipment required, and the technical constraints, including safety concerns in using a radioactive material. $^{51}\text{Cr-EDTA}$ is considered a relatively safe radionuclide for diagnostics in nuclear medicine for both the patient and the operator, as the radiation dose delivered to a patient after the oral administration of 3.7 MBq (100 µCi) of $^{51}\text{Cr-EDTA}$ is calculated to be less than 0.163 mSv (a dose that is far lower than any other nuclear diagnostic procedure and even six-fold lower than the radiation emitted during a routine abdominal radiograph). Moreover, the risk to operators handling the radioactive nuclide or administering the test solution containing $^{51}\text{Cr-EDTA}$ is considered negligible, providing that care is taken to avoid external contamination from vomit or urine spills from the canine patient. However, the use of radioisotopes requires expensive materials and equipment, and is restricted to legally-licensed operators and facilities. (ICRP, 1998, Hall, 1999, ICRP, 2002, ICRP, 2002)

Nevertheless, among all the positive and negative features of $^{51}\text{Cr-EDTA}$, the physiological and biochemical inertness of this molecule that makes it resistant against bacterial degradation, and the ease with which it can be quantified in urine using a γ-counter, makes it an especially valuable marker for intestinal permeability studies in comparison with sugars. Of paramount importance is the absolute resistance against bacterial degradation, because intestinal permeability testing is principally aimed at individuals who may potentially suffer from dysbiosis, including small intestinal bacterial overgrowth (SIBO), a condition that may be precipitated in patients with intestinal abnormalities and that may also be present in dogs as a subclinical entity in certain breeds such as Beagles. (Batt et al., 1992, Hall and Batt, 1996, Marks and Williams, 1998, Hall, 1999)

### 2.3.3 The dual sugar test

The simultaneous administration of two sugar molecules differing in size and weight, and the subsequent assessment of their differential urinary or blood recovery has been
more commonly used for permeability testing than the $^{51}$Cr-EDTA test. Results from the use of two markers for permeability testing are generally believed to be more sensitive than using a single probe, because the two molecules should be equally influenced by non-mucosal factors and the sources of error are considered to affect both probes equally. (Menzies, 1974, 1984, Bjarnason et al., 1988, Bjarnason and Peters, 1989, Hall and Batt, 1991c, Bjarnason et al., 1995, Hall, 1999)

In dogs, the differential permeability sugar test has typically been performed by combining a disaccharide such as lactulose (or cellobiose) and a monosaccharide such as rhamnose (or mannitol). The disaccharides lactulose and cellobiose are both rapidly metabolized by colonic bacteria, and their absorption theoretically reflects small intestinal permeability. The permeation of the monosaccharides rhamnose and mannitol is believed to occur throughout the crypt–villus axis in the small intestine, and their uptake reflects small intestinal permeability. An overall increased intestinal permeability is reflected by an increased disaccharide/monosaccharide ratio, which is explained by an increased recovery in the urine or blood of the larger molecule (i.e. disaccharide) coupled with a decreased recovery of the smaller one (i.e. monosaccharide). (Menzies, 1984, Hall and Batt, 1991c, Bjarnason et al., 1995, Hall, 1999, Arrieta et al., 2006)

The dual intestinal permeability test is frequently combined with sucrose, xylose, and 3-O-methyl-D-glucose. Sucrose, a monosaccharide that is immediately digested by the jejunal mucosa, has been used for the concurrent measurement of gastroduodenal permeability. Xylose and 3-O-methyl-D-glucose are monosaccharides that have been used for the measurement of small intestinal absorptive function. The simultaneous measurement of gastric permeability and the absorptive capacity of the small intestine usually provides complementary information on the gastrointestinal tract, which can be useful for estimation of the overall severity of mucosal damage. (Menzies, 1974, Elwood et al., 1993, Bjarnason et al., 1995, Meddings et al., 1995, Rutgers et al., 1995, Rutgers et al., 1996, Hall, 1999, Suchodolski and Steiner, 2003, Batt, 2009)

The canine differential sugar test was first described as a urine test using cellobiose and mannitol as permeability markers (Hall and Batt, 1991c), but the lactulose and rhamnose test has become the standard sugar intestinal permeability test in dogs. Although the analytical procedure is technically more demanding for lactulose and rhamnose than for cellobiose and mannitol, the use of the latter combination has been discouraged because there is some sugar hydrolysis caused by small intestinal cellobiose activity of brush-border enzymes, and also because endogenous mannitol has been detected in dogs and in the urine of fasted subjects. (Dahlqvist, 1962, Laker et al., 1982, Noone et al., 1986, Hall and Batt, 1991c, Elwood et al., 1993, Quigg et al., 1993, Bjarnason et al., 1995, Hall and Batt, 1996)

The lactulose/rhamnose test, and more recently the lactulose/mannitol assay, has been adapted for blood methods in dogs. The blood approach is more practical than the urine-based test during the testing procedure in dogs, since collecting a blood sample between 90 and 180 minutes after dosing (Sørensen et al., 1997, Rodríguez et al., 2009)
is more advantageous than the cumulative recovery of urine over a period of at least 5 hours and sometimes up to 24 hours. However, protocols utilizing permeability markers recovered in urine have been more commonly used than blood methods, mainly because the quantification of sugars in serum or plasma is more laborious and time consuming than in urine. Although the determination of saccharides in the urine and blood of dogs has been improved and has made testing simpler than previous methodologies used in the past, the detection of sugars in blood is more cumbersome than in urine, essentially because the blood sample must be deproteinized and the endogenous glucose must be oxidized. (Sørensen et al., 1993, Sørensen et al., 1997, Hall, 1999, Steiner et al., 2000, Rodriguez et al., 2009a, Rodriguez et al., 2009b)

The measurement of di- and monosaccharides used in permeability screening is performed by high-performance liquid chromatography. More recently, a gas chromatography–mass spectrometry (GC-MS) method for the quantification of lactulose, rhamnose, xylose, 3-O-methylglucose, and sucrose in canine serum has been developed and analytically validated. This method was shown to be accurate, precise, and reproducible for the simultaneous measurement of sugar probes in canine serum. (Rodriguez et al., 2009b, Rodríguez et al., 2009)

The use of saccharides for assessing intestinal permeability has been associated with inconsistent results (Allenspach et al., 2006, Kobayashi et al., 2007), and such inaccuracies have been ascribed to some of the physicochemical properties of the saccharides. Although widely claimed as inert molecules, sugars are subject to intestinal hydrolization and bacterial degradation, phenomena that can considerably affect the recovery of the markers, especially in patients with bacterial overgrowth or dysbiosis. (Elia et al., 1987, Katz and Hollander, 1989) This circumstance has largely been overlooked because non-mucosal factors such as bacterial metabolism of probes would not affect test results, as these are expressed as the ratio of two molecules. However, it is well known that the rate of bacterial metabolism for each of the two sugars is not necessarily identical, so the ratio of two molecules may not necessarily provide accurate permeability test results. (Laker et al., 1982, Noone et al., 1986, Quigg et al., 1993, Riordan et al., 1997, Garden et al., 1998) Similarly, it is known that the urinary excretion of the saccharides used as permeability probes has been unequal and incomplete 24 hours after intravenous administration in clinically healthy dogs (e.g. rhamnose 72%) (Hall and Batt, 1996, Garden et al., 1998), phenomena that could also lead to errors in the data generated from intestinal permeability tests using such sugars.

In humans, it has been reported, however, that the sensitivity of the two-sugar test is low and inadequate in patients that have SIBO with an overgrowth primarily composed of colonic-type bacteria. (Riordan et al., 1997) In dogs, different rates of sugar metabolism may lead to variations in the lactulose/rhamnose ratios, a process that may be further aggravated in subjects with SIBO. (Hall and Batt, 1996, Hall, 1999)
The lactulose/rhamnose intestinal permeability test in blood for dogs has been developed (Sørensen et al., 1993, Sørensen et al., 1997, Rodriguez et al., 2009b), but because the analysis of sugars in plasma or serum is technically demanding, this blood approach has not gained widespread use in veterinary medicine.

### 2.3.4 Contrast media for permeability testing

Non-ionic water-soluble iodinated radiographic contrast media of low- and iso-osmolar types, such as iohexol and iodixanol, are widely used in human and small animal medicine for X-ray-based imaging techniques such as radiography and computed tomography (Wood et al., 1985, Kishimoto et al., 2010), and for assessing the glomerular filtration rate (Moe and Heiene, 1995, Gleadhill and Michell, 1996). These agents have also recently been identified as valuable molecules for measuring intestinal mucosal damage in humans and rats.

Iohexol and iodixanol agents were selected because they share many physicochemical features required for permeability probes, including an appropriate molecular size and mass, hydrophilicity, intestinal uptake by passive diffusion, non-reactivity, non-toxicity, metabolic stability, and easy quantification after urine excretion. (Andersen and Laerum, 1995)

In particular, the 24-h excretion rates of iohexol in urine after IV application have been reported to be 99% for humans, 96.1 ± 4.7% for dogs (98 ± 4% after 7 days), and 91.5 ± 3.6% for rats (93–95% after 7 days). In addition, the urine specimens in these species did not reveal any metabolized forms of iohexol, so it has been concluded that iohexol is excreted unchanged in the urine of these species after intravenous application. Moreover, iohexol is a substance that may be detected in canine plasma after oral administration to dogs. As a radiocontrast agent, it is frequently available in the departments of veterinary radiology, and it has been successfully used as a preclinical marker of intestinal damage in experimental rats, and clinically in human patients. (Aakhus et al., 1980, Mützel and Speck, 1980, Stordahl, 1989a, Solheim et al., 1991, Andersen et al., 1992, Halme, 1992, Halme et al., 1993, Agut et al., 1995, Gaspari et al., 1995, Halme et al., 1997, Halme et al., 2000, Andersen et al., 2001, Finco et al., 2001, Gerova et al., 2011)

In contrast to the above-described intestinal permeability markers used in dogs, iohexol is non-radioactive, is not inconsistently degraded by intestinal bacteria, and has more potential applications as it may be simultaneously used, for example, in the radiographic examination of intestinal morphology by X-ray fluorescence and possibly also computed tomography densitometry. (Grönberg et al., 1983, Stordahl, 1989b, Lundqvist et al., 1995, Rencken et al., 1997)
3. **AIMS OF THE STUDY**

The main objective of this work was to improve fundamental aspects of intestinal permeability testing in dogs in order to make the screening of intestinal mucosal damage A) more practical and accurate, and B) more attractive and accessible for the non-invasive investigation of canine intestinal and extra-intestinal diseases in veterinary clinical and biomedical research. Preclinical studies using laboratory Beagle dogs or Sprague-Dawley rats were performed, and the specific aims of these studies were:

1. To evaluate and compare the most relevant markers used in intestinal permeability testing in dogs. Specifically, the gold standard probe $^{51}$Cr-EDTA and the most commonly used probes lactulose and rhamnose were simultaneously used in healthy laboratory Beagles, in order to elucidate whether the use of $^{51}$Cr-EDTA and such sugars are truly comparable for the measurement of intestinal permeability in dogs.

2. To determine the relevance of using a single marker such as $^{51}$Cr-EDTA or lactulose, or a combination of two as a ratio, such as $^{51}$Cr-EDTA/rhamnose or lactulose/rhamnose, for the measurement of intestinal permeability in dogs.

3. To determine the reliability of intestinal permeability blood tests in dogs in comparison with the urine-based test.

4. To compare iohexol and the gold standard marker $^{51}$Cr-EDTA for the assessment of intestinal permeability in healthy laboratory Beagle dogs.

5. To pre-clinically evaluate iohexol as an intestinal permeability marker in diseased subjects using a well-characterized laboratory murine model of intestinal disease.
4. MATERIALS AND METHODS

4.1 Experimental animals

A total of 33 laboratory Beagle dogs and 30 Sprague-Dawley rats were used in the studies (Table 5).

Table 5. Characteristics of the animals used in the studies.

<table>
<thead>
<tr>
<th>Species and breed/strain</th>
<th>Study</th>
<th>Number (and sex)</th>
<th>Age</th>
<th>Weight</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beagle dogs</td>
<td>I</td>
<td>19 (♂)</td>
<td>11–40 mo.</td>
<td>12.4–17 kg</td>
<td>Healthy, no gastrointestinal (GI) signs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mean 18)</td>
<td>(mean 14.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>31 (29♂, 2♀)</td>
<td>1–5 yrs.</td>
<td>10.3–17.8 kg</td>
<td>Healthy, no GI signs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mean 1.9)</td>
<td>(mean 15.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>13♂</td>
<td>17–46 mo.</td>
<td>13–17 kg</td>
<td>Healthy, no GI signs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mean 23.1)</td>
<td>(mean 15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>7 (5♂, 2♀)</td>
<td>3–7 yrs.</td>
<td>10.4–19.5 kg</td>
<td>Healthy, no GI signs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mean 5.4)</td>
<td>(mean 14)</td>
<td></td>
</tr>
<tr>
<td>Hsd:Sprague Dawley®™SD®™ rats</td>
<td>V</td>
<td>30 (28♀)</td>
<td>Two were excluded because of esophageal reflux.</td>
<td>12–13 wks.</td>
<td>200–250 g</td>
</tr>
</tbody>
</table>

The experimental protocols using dogs (Studies I, II, III, IV) were approved by the local Ethics Committee for Animal Experiments of the University of Helsinki, Finland. The project using rats (Study V) was approved by the local Ethics Committee for Animal Experiments of the University of Turku, Finland.

All the dogs and rats were cared for and used in experiments in accordance with the principles outlined in the prevailing Finnish and European legislation on the use of vertebrate animals for scientific purposes (European Community Council Directive 86/609/EEC, Council of Europe, 1986; Finnish Government, 1985; Finnish Government, 1996).

4.1.1 Dogs

All the Beagles used in the studies were purebred laboratory dogs that were supplied by approved laboratory animal breeders (Harlan-Winkelmann GmbH, Borchen, Germany; and the National Laboratory Animal Center, University of Kuopio, Finland). All the dogs were acclimatized for at least one month prior to the start of the experiments, and they were housed in the former experimental dog facilities of the Faculty of Veterinary
Materials and Methods

Medicine, University of Helsinki, Finland. The dogs were maintained in indoor pens, spending about four hours daily in outdoor runs, and were exposed to both natural and artificial light (from 07:00 to 16:00). The environmental temperature indoors was maintained within a range of approximately 15–24 °C. Feeding throughout the studies consisted of 1.5 cans/dog of a commercial canned dog food (Pedigree®, Fortivil 400 g, Waltham®, Masterfoods Ltd, Helsinki, Finland), which was given twice daily. Water was freely available at all times.

None of the dogs were considered obese based on visual observations, and all the dogs were determined to be healthy based on results from physical examinations, and after interpretation of hematological and serum biochemical analysis. Blood urea nitrogen and serum creatinine concentrations suggested normal renal function in all dogs. Exocrine pancreatic function was considered normal based on measurements of serum trypsin-like immunoreactivity concentrations (Williams and Batt, 1988). Serum folate and cobalamin concentrations suggested normal carrier-mediated absorption in the proximal and distal small intestine (Batt and Morgan, 1982).

A few days prior to commencing the intestinal permeability testing, the dogs were dewormed with fenbendazole (Axilur®, Intervet International, Boxmeer, The Netherlands) at a dose of 50 mg/kg PO for 3 consecutive days. Subsequent examination of fecal samples for endoparasitic ova was negative for all dogs.

4.1.2 Rats

All the Hsd:Sprague Dawley®™SD®™ (SD) rats used in study V were obtained from a breeding colony supplied by a semi-barrier facility of the Central Animal Laboratory, University of Turku, Finland.

Prior to the commencement of the study, the rats enrolled in the study were acclimatized for 21 days and were determined to be healthy on the basis of individual physical examinations, and specific pathogen-free based on the results of routine microbiological screening performed on the rat colony in accordance with European recommendations. (Nicklas et al., 2002)

At the commencement of the study, the rats were 12 weeks old and ranged in body weight from 200 to 250 g. The rodents were housed in groups of six and were maintained in opened stainless steel cages (59.5 x 38.0 x 20.0 cm) with solid bottoms and Aspen chips used as bedding (Tapvei Ltd, Kaavi, Finland). An Iglo and some nesting material were used as enrichment. Cage change was undertaken twice a week. The environment in the room was maintained at an approximate temperature of 22 °C (range 20 to 23 °C), a relative humidity of 50 to 60%, and artificial illumination with a 12-h light/dark cycle (lights on at 06:00 am). Throughout the study period, all the rats were fed a standard rat chow (SDS, Special Diet Services, Whitham, Essex, UK) ad libitum, and tap water was provided without restrictions in polycarbonate bottles.
Gastrointestinal damage was induced by a 7-day administration of 5% dextran sulfate sodium (DSS) in drinking water, which has been shown to produce symptoms in laboratory rats comparable to inflammatory bowel disease (IBD) observed in humans. After exposure to DSS, the rats developed the typical clinical symptomatology associated with inflammatory bowel disease, and all the subjects were determined to be affected based on physical examination and evidence of changes in fecal consistency, diarrhea and hematochezia. (Gaudio et al., 1999, Chen et al., 2007)

To assess the possible toxic effects of DSS on kidney function, creatinine concentrations were determined in urine samples using a Konelab 30i automatic analyzer (Thermo Scientific, Waltham, MA, USA). The iohexol-to-creatinine ratio was also calculated similarly to the urinary protein-to-creatinine ratio for the assessment of proteinuria in dogs. (White et al., 1984, Grauer et al., 1985) The results from the serum creatinine concentrations suggested normal renal function before and after exposure to DSS.

4.2 Permeability testing in dogs

Before intestinal permeability testing was carried out in dogs (I, II, III, IV), their food was withheld the night before and throughout the study, but water was freely available at all times. Following the overnight fasting period and immediately prior the administration of the permeability markers delivered in the test solutions, the body weights of the dogs were measured and a baseline blood sample was collected from each subject.

On the same morning of the experiments, fresh individual test solutions were made by dissolving the probe molecules with distilled water. All the dogs received the test solutions intragastrically by using an orogastric tube. If esophageal reflux or fecal contamination of urine was observed, the test was cancelled and repeated after an interval of at least five days (Hall et al., 1989).

In studies using $^{51}$Cr-EDTA as permeability probe (I–IV), individual test solutions consisted of approximately 3.7 MBq (100 µCi) of the radioisotope (Nycomed Amersham plc, Little Chalfont, Buckinghamshire, United Kingdom) dissolved in 50 ml of distilled water. A counting standard from the test solution was retained in a 1-ml aliquot, which was further diluted (1:50) before radioactivity measurements.

In study I, two test solutions were administered. The solution containing $^{51}$Cr-EDTA was administered first, and then immediately followed by the administration of 200 mL of distilled water containing a mixture of 2 g lactulose (L), 2 g rhamnose (R), 2 g D-xylose (X), 1 g 3-O-methyl-D-glucose (G), and 8 g sucrose (S).

In study II, the $^{51}$Cr-EDTA test was simultaneously performed on urine and serum in the same dogs (n = 31). The testing was performed a total of 43 times, once in 25 dogs and thrice in 6 dogs.
In study III, the $^{51}$Cr-EDTA permeability test in blood was simultaneously performed on serum and plasma. Two concurrent blood samples were withdrawn from the cephalic veins of each dog at 3 and 5 h post-administration of the test solution. In this study, one of the blood samples was transferred to clotting factor activator tubes to obtain serum, and the other one was transferred to heparinized tubes to obtain plasma. All the blood samples were centrifuged for 12 min at 2100 $g$ to obtain either serum or plasma. The sera and plasma were separated into 1- to 2-ml aliquots for the measurement of radioactivity.

In study IV, a single dose of 10 mL of Omnipaque 300 (Amersham Health) containing 6471 mg of iohexol was added to a test solution containing $^{51}$Cr-EDTA dissolved in 50 mL of distilled water.

In studies I and II, all of the dogs were sedated with medetomidine (Domitor®, Orion Pharma Ltd, Turku, Finland), 25 µg/kg IM, just prior to the administration of the test solution and to facilitate the emptying of the urinary bladders via catheterization. During the mild sedative state of the dogs, they received the test solutions by orogastric gavage, and subsequently after that atipamezole (Antisedan®, Orion Pharma Ltd, Turku, Finland), 100 µg/kg IM, was injected to reverse the sedative effects of medetomidine. The dogs were then placed in metabolic cages for 6 hours for urine collection, and at the end of this period medetomidine at 25 µg/kg was once again injected IM to each dog prior to catheterization of the urinary bladders for urine collection. All the urine collected from the urinary bladders and from the metabolic cages was pooled, and the total urine volume was determined and recorded for later test calculations for each dog. In studies III and IV, no sedative drugs were used.

In studies I and II, a 2-ml urine aliquot from the total urine volume was retained for the measurement of $^{51}$Cr-EDTA radioactivity. In study I, a further 2-ml tube was retained for analysis of sugars, which was first stored at -20 °C and subsequently shipped on dry ice to the GI Laboratory, Texas A&M University, College Station, Texas, USA, for the analytical procedure.

In studies II and IV, all timed blood samples were withdrawn from cephalic veins post-administration of the test solution (study II at 2, 3, 4, 5, and 6 hours; study IV at 0.5, 1, 2, 3, 4, 5, and 6 hours).

### 4.3 Permeability testing in rats

One mL of Omnipaque 300® (iohexol, 647.1 mg/mL) was dosed intragastrically to each rat using a feeding needle. No sedative drug was used before, during or after administration. The animals were placed in individual metabolic cages for urine collection during 24 hours. After all urine had been recovered, the volumes were recorded and the samples frozen at -18 °C until later analysis. If esophageal reflux of iohexol or fecal contamination of urine was observed, the test was cancelled.
4.4 Measurement of permeability markers

All the control, urine, serum, and plasma aliquots were measured for $^{51}$Cr-EDTA gamma ray emissions by use of a counter (LKB-Wallac 1270 Rackgamma II gamma counter, LKB-Wallac, Turku, Finland). Gamma ray emissions in the aliquots were counted for 10 min, and all the quantifications were performed during the same evening of the experiments and within 12 hours of the end of the collection period for each animal at the former facilities of the Central Laboratory of the Department of Clinical Veterinary Sciences, University of Helsinki, Finland.

The analysis of sugars in canine urine (study I) was performed by high-performance liquid chromatography in accordance with a previously reported methodology (Steiner et al., 2000).

The iohexol concentration in the urine of rats (study V) and serum of dogs (study IV) was analyzed by high-performance liquid chromatography with ultraviolet detection [(HPLC)-UV] after solid phase extraction, as previously described elsewhere. (Klenner et al., 2007, Pöytäkangas et al., 2010)

4.5 Formulas to calculate the recovery rates of $^{51}$Cr-EDTA and iohexol.

The amount of $^{51}$Cr-EDTA in urine, serum, and plasma (I–IV) was calculated as a percentage of the orally-ingested test solution using the formulas in Table 6.

The amount of iohexol in rat urine and dog serum as a percentage of the orally-ingested test dose was calculated using the formulas in Table 7.

Table 6. Formulas to calculate the amount of $^{51}$Cr-EDTA as a percentage of the orally-ingested test solution in canine urine, serum, and plasma. The total blood (TBV), serum (TSV), and plasma (TPV) volumes were estimated according to formulas published elsewhere. (Woodward et al., 1968, Jain, 1986). Modified from studies II and III.

$^{51}$Cr-EDTA in urine (%) = \( \frac{\text{cpm in urine aliquot (1 mL) x TUV (mL)}}{\text{cpm in standard aliquot (1 mL) x 50 (DF) x TTSV (mL)}} \times 100 \)

$^{51}$Cr-EDTA in serum (%) = \( \frac{\text{cpm in serum aliquot (1 mL) x TSV (mL)}}{\text{cpm in standard aliquot (1 mL) x 50 (DF) x TTSV (mL)}} \times 100 \)

$^{51}$Cr-EDTA in plasma (%) = \( \frac{\text{cpm in serum aliquot (1 mL) x TPV (mL)}}{\text{cpm in standard aliquot (1 mL) x 50 (DF) x TTSV (mL)}} \times 100 \)

TBV (mL) = BW (kg) x 100 (mL/kg)

TSV and TPV = \( \frac{\text{TBV (mL) x (100 – H)}}{100} \)

Legend: cpm, counts per minute; TUV, total urine volume; TBV, total blood volume; TSV, total serum volume; TPV, total plasma volume; TTSV, total test solution volume; TPV, total plasma volume; DF, dilution factor; BW, body weight; H, hematocrit
Materials and Methods

Table 7. Formulas to calculate the amount of iohexol as a percentage of the orally-ingested test solution in rat urine and dog serum. Modified from studies IV and V.

\[
\text{Iohexol in rat urine} (\%) = \frac{\mu g/mL \text{ in urine} \times TUV (mL)}{\mu g \text{ in test solution} (1 \text{ mL Omnipaque 300})} \times 100
\]

\[
\text{Iohexol in dog serum} (\%) = \frac{\mu g/mL \text{ in serum} \times TSV (mL)}{\mu g \text{ in test solution} (10 \text{ mL Omnipaque 300})} \times 100
\]

Legend: TUV, total urine volume; TSV, total serum volume.

4.6 Statistical analysis (I–V)

The following statistical analyses were used in the original articles.

I. The Shapiro-Wilk test was used to assess the normality of the distribution of each variable. Normally distributed variables were expressed as means (± SD, range), and other variables as medians (range). Pearson’s correlation coefficient was used to measure the associations between pairs of probe markers that both followed a normal distribution, whereas Spearman’s rank correlation coefficients were calculated if one of the variables was not normally distributed. Values of \( P \) less than 0.05 were considered significant. Statistical analysis was performed using a commercial software program (SAS system for Windows, release 9.2, SAS Institute, Cary, NC, USA).

II. Results were expressed as mean ± SD. Coefficients of variation are presented as percentages (CV %). The relationship between the results in urine and blood was determined using the Pearson correlation coefficient (r), which was calculated using statistical analysis tools from a software program (Microsoft® Excel 2002, ©Microsoft Corporation 1985-2001).

III. Statistical analysis was performed using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). The data distribution was analyzed by applying the Kolmogorov–Smirnov test and was determined to be Gaussian. Due to the normal distribution, results were expressed as the mean and standard deviation (mean ± SD). The significance of the difference in test results between serum and plasma samples was determined by one-way analysis of variance, and where appropriate, the Student–Newman–Keuls test was used for the comparison of means. The relationship between the results was determined using the Pearson correlation coefficient (R). A P-value of less than 0.05 was regarded as statistically significant. The Bland–Altman test was used to analyze the difference and potential bias between the two methods. The repeatability coefficient was calculated as 1.96 times the SD of the differences.

IV. The MedCalc software program (v. 11.2.1.0) was used for statistical analysis. According to the Shapiro-Wilk test, serum concentrations of \(^{51}\text{Cr-EDTA and}

...
iohexol followed a normal distribution. Longitudinal variables were considered independent, and the data were processed using a simple linear regression model, correlation analysis and paired-samples t-test. $P < 0.05$ was considered significant.

V. Statistical analysis was performed using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). The data were analyzed with the Wilcoxon signed-ranks test, and were expressed as the median (IQR).
5. RESULTS

5.1 Urinary measurements and statistical comparisons of the markers after their concurrent oral administration to healthy adult male Beagles (I)

The individual age and weight of the nineteen healthy male laboratory Beagles used in study I, and the percentage urinary recovery of the 6 markers used in this study to assess gastrointestinal permeability and absorptive function after their simultaneous orogastric administration are presented in Table 8.

The urinary excretion of $^{51}$Cr-EDTA ($P = 0.155$), lactulose ($P = 0.331$), rhamnose ($P = 0.388$), the ratio of $^{51}$Cr-EDTA/rhamnose ($P = 0.077$), lactulose/rhamnose ($P = 0.062$), 3-O-methyl-D-glucose ($P = 0.121$), and the ratio of D-xylose/3-O-methyl-D-glucose ($P = 0.181$) were normally distributed. However, D-xylose ($P = 0.035$) and sucrose ($P = 0.000$) did not follow a normal distribution.

The mean ($\pm$ SD, range) percentage urinary recovery of intestinal permeability markers $^{51}$Cr-EDTA, lactulose, and rhamnose was 6.3% ($\pm$ 1.6%, 4.3–9.7%), 3.3% ($\pm$ 1.1%, 1.7–5.3%), and 25.5% ($\pm$ 5.0%, 16.7–36.9%), respectively. The median (range) percentage urinary recovery of the intestinal absorptive function marker D-xylose was 40.3% (31.6–62.7%), and the mean ($\pm$ SD, range) percentage urinary recovery of 3-O-methyl-D-glucose was 58.8% ($\pm$ 11.0%, 40.1–87.8%). The median (range) percentage urinary recovery for sucrose (gastric permeability marker) was 0.0% (0.0–0.8%).

The mean ($\pm$ SD, range) urinary recovery ratio was 0.25 ($\pm$ 0.06, 0.17–0.37) for $^{51}$Cr-EDTA/rhamnose, 0.13 ($\pm$ 0.04, 0.08–0.23) for lactulose/rhamnose, and 0.73 ($\pm$ 0.09, 0.60–0.90) for D-xylose /3-O-methyl-D-glucose.

The correlation coefficients and significance levels for the biomarkers evaluated in study I are presented in Table 9.
Table 8. Individual data on healthy male laboratory Beagles (study I), and the recovery of markers in urine after orogastric administration.

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>$^{51}$Cr-EDTA % recovery</th>
<th>L % recovery</th>
<th>R % recovery</th>
<th>$^{51}$Cr-EDTA/R recovery ratio</th>
<th>L/R recovery ratio</th>
<th>X % recovery</th>
<th>G % recovery</th>
<th>X/G recovery ratio</th>
<th>S % recovery</th>
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<td>1</td>
<td>4.7</td>
<td>2.1</td>
<td>25.2</td>
<td>0.19</td>
<td>0.08</td>
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<td>0</td>
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<td>2</td>
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<td>2.7</td>
<td>22.9</td>
<td>0.27</td>
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<td>34.8</td>
<td>50.5</td>
<td>0.69</td>
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<td>0.26</td>
<td>0.13</td>
<td>34.1</td>
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Table 9. Correlation coefficients and their significance levels (P values) between the urinary percentage recoveries for the biomarkers. Modified from study I.

<table>
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<tr>
<th></th>
<th>Lactulose</th>
<th>Rhamnose</th>
<th>$^{51}$Cr-EDTA/R</th>
<th>L/R</th>
<th>Xylose</th>
<th>3-O-methyl-D-glucose</th>
<th>X/G</th>
<th>Sucrose</th>
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<td>$^{51}$Cr-EDTA</td>
<td>0.64**</td>
<td>0.40</td>
<td>0.73***</td>
<td>0.51*</td>
<td>-0.07</td>
<td>0.16</td>
<td>-0.47*</td>
<td>0.00</td>
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<td>Rhamnose</td>
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<td>0.20</td>
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<td>0.37</td>
<td>0.61***</td>
<td>0.90***</td>
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<tr>
<td>$^{51}$Cr-EDTA/R</td>
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<td>0.63***</td>
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Correlations were calculated using Pearson’s or Spearman’s rank coefficients where appropriate. Significance levels are expressed as: *P < 0.05, **P < 0.01, and ***P < 0.001.
5.2 Comparison between the $^{51}$Cr-EDTA test measured in blood and urine in healthy adult Beagles (II)

The mean levels of $^{51}$Cr-EDTA in serum progressively increased until reaching a peak, and thereafter declined gradually (Fig. 6). The peak of $^{51}$Cr-EDTA in serum took place within 5 hours after its oral administration in 41 of the 43 trials attempted (95%).

The mean ± SD (range) percentage of the orally-administered $^{51}$Cr-EDTA in all the observations (n = 43) in urine after 6 hours was 14.07 ± 8.72% (3.81–34.18%), while percentage recoveries in serum from the blood samples taken at 2, 3, 4, 5, and 6 hours were 0.49 ± 0.45% (0.02–2.13%), 0.75 ± 0.52% (0.03–1.89%), 0.82 ± 0.57% (0.13–2.21%), 0.70 ± 0.53% (0.12–1.99%), and 0.47 ± 0.44% (0.11–1.79%), respectively.

The correlation coefficients between urine and the serum samples (n = 43) are presented in Table 10. The results indicate that better correlations were obtained by summing the recovery rates for multiple blood samples. Although excellent correlations were obtained by combining the results for only two blood samples taken at 3 and 5 hours ($r = 0.95$) or at 3 and 4 hours ($r = 0.94$), the sum of recoveries for 4 or more blood samples gave the best correlations with the urinary recovery ($r = 0.97$) after oral administration of the test solution. Furthermore, in the 6 dogs that underwent repeated testing, the correlation coefficients between urine and individual blood samples varied from -0.01 to 0.91. Likewise, the combination of 4 and more blood samples produced correlation

![Figure 6](image-url) Profile of the mean course of $^{51}$Cr-EDTA activity in serum during 6 h after its oral administration to dogs (n = 43).

**Table 10.** Correlation coefficients between urine and serum samples (n = 43) of percentage recoveries of $^{51}$Cr-EDTA after its oral administration to Beagles. Modified from study II.

<table>
<thead>
<tr>
<th>% URINE (0-6h)</th>
<th>% SERUM</th>
<th>% SERUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficients</td>
<td>Single samples</td>
<td>Two samples</td>
</tr>
<tr>
<td>2 h</td>
<td>3 h</td>
<td>4 h</td>
</tr>
<tr>
<td>0.61</td>
<td>0.84</td>
<td>0.89</td>
</tr>
</tbody>
</table>
coefficients that varied from 0.97 to 0.99, whereas with the combination of only two blood samples taken at 3 and 5, and at 3 and 4 hours, the correlation coefficient varied from 0.91 to 0.98.

5.3 Comparison between the $^{51}$Cr-EDTA blood test measured in serum and plasma in healthy adult Beagles (III)

The mean ± SD percentages of $^{51}$Cr-EDTA in serum and plasma after 3 h were 0.85 ± 0.43% and 0.88 ± 0.49%, respectively, whereas the respective percentages in serum and plasma after 5 h were 0.78 ± 0.52% and 0.81 ± 0.51%. No significant differences were detected between the percentages of $^{51}$Cr-EDTA in serum and plasma samples at 3 and 5 h (Fig. 7). In addition, statistically significant correlations were found between serum and plasma levels of $^{51}$Cr-EDTA at 3 h ($R = 0.96, P < 0.0001$), and at 5 h ($R = 0.99, P < 0.0001$; Fig. 8). The combined correlation coefficient between the percentages from the serum and plasma samples was excellent ($R = 0.98$).

![Figure 7. $^{51}$Cr-EDTA recovery percentages from serum (grey) and plasma (white) samples at 3 and 5 h after ingestion. The horizontal line in the box is the median (50% percentile), and the upper and lower limits of the box indicate the 75% upper and 25% lower quartiles, respectively. The limits of the upper and lower vertical lines represent the maximum and minimum data values, respectively. No outliers were detected in the samples. Reprinted with permission from study III.](image-url)
Figure 8. Relationships between serum and plasma levels at 3 h (□ and discontinuous line) and 5 h (● and continuous line) after ingestion of $^{51}$Cr-EDTA dissolved in water. Lines show the Pearson correlation (linear adjustment) at 3 h (R = 0.96, P < 0.0001, and at 5 h (R = 0.99, P < 0.0001). Reprinted with permission from study III.

5.4 Comparison between $^{51}$Cr-EDTA and iohexol as permeability blood markers in healthy adult Beagles (IV)

The mean percentage recoveries in serum of $^{51}$Cr-EDTA and iohexol over the 6-h time course after simultaneous oral administration of both markers, and the significance of the difference in serum recovery between the two markers at each time point is shown in Table 11.

Figure 9 illustrates the mean (± SD) percentage recoveries of $^{51}$Cr-EDTA and iohexol at each time point, whereas Figure 10 displays the positive linear association between $^{51}$Cr-EDTA and iohexol after analysis of all serum values from all dogs throughout the 6-h time course.
Table 11. $^{51}$Cr-EDTA and iohexol mean recovery percentages in serum samples of seven healthy Beagle dogs after oral administration, and differences between both markers during the 6-h time course. Reprinted with permission from study IV.

<table>
<thead>
<tr>
<th>Time Hours</th>
<th>$^{51}$Cr-EDTA (%) Mean</th>
<th>Iohexol (%) Mean</th>
<th>Difference between $^{51}$Cr-EDTA and Iohexol 95% CI</th>
<th>P-value paired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.266</td>
<td>0.032</td>
<td>0.23</td>
<td>0.13-0.33</td>
</tr>
<tr>
<td>1</td>
<td>0.306</td>
<td>0.048</td>
<td>0.26</td>
<td>0.18-0.34</td>
</tr>
<tr>
<td>2</td>
<td>0.282</td>
<td>0.048</td>
<td>0.23</td>
<td>0.14-0.33</td>
</tr>
<tr>
<td>3</td>
<td>0.182</td>
<td>0.033</td>
<td>0.15</td>
<td>0.05-0.11</td>
</tr>
<tr>
<td>4</td>
<td>0.098</td>
<td>0.022</td>
<td>0.08</td>
<td>0.03-0.12</td>
</tr>
<tr>
<td>5</td>
<td>0.067</td>
<td>0.013</td>
<td>0.06</td>
<td>0.03-0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.059</td>
<td>0.011</td>
<td>0.05</td>
<td>0.02-0.07</td>
</tr>
</tbody>
</table>

Figure 9. Time course and percentage recovery in serum of $^{51}$Cr-EDTA and iohexol expressed as mean values ± SD after oral administration of both markers to seven healthy Beagle dogs. In A) the curves for both markers are shown on the same scale; in B) the curve for iohexol has been re-scaled to aid in the visual comparison with $^{51}$Cr-EDTA. Reprinted with permission from study IV.
Figure 10. Linear association (correlation and simple regression equation model) between the percentage recovery in serum of $^{51}$Cr-EDTA and iohexol at seven time points (between 0.5 and 6 h) from all seven dogs. Discontinuous lines represent the 95% confidence intervals. Reprinted with permission from study IV.

5.5 Evaluation of iohexol as a permeability marker in healthy rats and in rats with DSS-induced inflammatory bowel disease (V)

The median percentage (%) (IQR) of iohexol in healthy rats was 0.54% (0.36–0.75%), whereas the respective value after DSS administration was 11.42% (5.58–15.37%). The median (IQR) iohexol/creatinine ratio was 0.05 (0.03–0.06) in healthy rats and 1.38 (0.76–2.49) in rats with IBD. Nonparametric comparison of the urinary excretion of iohexol as well as the iohexol/creatinine ratio demonstrated statistically significant differences ($P < 0.001$) between healthy rats and those with ulcerative colitis.
Figure 11. Percentile plots of urinary iohexol before and after the induction of inflammatory bowel disease by adding 5% DSS to the drinking water of SD rats (n = 28) for seven days. The line in the box represents the median (50%); the lower line represents the 25% lower quartile, and the upper line represents the 75% upper quartile. The limits of the upper and lower vertical lines indicate the maximum and minimum data values, respectively. The separate asterisks indicate outliers. Modified with permission from study V.
6. DISCUSSION

6.1 Reference ranges and comparisons between the most relevant permeability markers in healthy adult Beagles (I)

Because it is difficult to define an effective normal range of intestinal permeability that is common for all canine patients and breeds in both the veterinary clinical and biomedical research arenas, it has been necessary to determine a normal intestinal permeability index that is specific for each dog breed (Randell et al., 2001, Weber et al., 2002). The novelty of this research was to report the 6-h percentage urinary recovery after oral administration of $^{51}$Cr-EDTA, lactulose, rhamnose, D-xylose, 3-O-methyl-D-glucose, and sucrose in healthy adult male Beagle dogs housed in controlled laboratory conditions. It is well known that intrinsic differences exist between species in intestinal permeability related to the physical properties of the epithelium (Randell et al., 2001). Moreover, it has been determined that animal factors including breed, age, body size, sexual and health status, as well as environmental factors such as diet should be taken into account when defining normative ranges of intestinal permeability markers (Weber et al., 2002). However, these factors have largely been ignored in clinical practice and scientific reports using intestinal permeability tests in dogs. The overlooking of all such factors can certainly account for some of the variability observed in the normal recovery intervals for the markers in this species, and for the disparity observed in the results of some reports using permeability tests in dogs with enteropathies. This variability may be explained by the unconcerned use of pubertal and adult male and female dogs (intact and sterilized), which have been fed with different diet types (e.g. canned, dried or home-made diets), and by using apparently healthy dogs that could have been affected by subclinical intestinal disease or small intestinal bacterial overgrowth, as has been reported for Beagle dogs and humans affected with coeliac disease. (Delahunty and Hollander, 1987, Batt et al., 1992, Jezyk et al., 1992, Ferguson et al., 1993, Bijlsma et al., 1995, Kararli, 1995, Garden et al., 1997, Garden et al., 1998, Vaden et al., 2000, Randell et al., 2001, Weber et al., 2002, Allenspach et al., 2006, Kobayashi et al., 2007, Berghoff, 2011)

In our studies using different intestinal permeability markers given simultaneously, the 6-h percentage urinary recovery for $^{51}$Cr-EDTA in healthy adult male Beagle dogs ranged from 4.3 to 9.7%, whereas the mean 24-h percentage urinary recovery of the radiolabeled chelate in clinically health dogs was previously reported to vary between 2.3 and 17.3% (Hall et al., 1989). The 6-h urinary recovery ratio between lactulose and rhamnose in the healthy adult male Beagle dogs of our studies ranged between 0.08 and 0.23. Reported results using healthy dogs of different breeds have shown the healthy ranges of lactulose/rhamnose to vary from 0.03 (Garden et al., 1997) to 0.42 (Weber et al., 2002), including 0.03–0.18 in Irish Setters (Garden et al., 1997), 0.19–0.34 in Greyhounds (Randell et al., 2001), 0.07–0.26 in Viszlas (Randell et al., 2001), 0.08–0.34
in mixed-breed dogs (Randell et al., 2001), 0.14–0.21 in adult Miniature Poodles (Weber et al., 2002), 0.13–0.26 in adult Standard Schnauzers (Weber et al., 2002), 0.17–0.32 in adult Giant Schnauzers (Weber et al., 2002), 0.26–0.42 in adult Great Danes (Weber et al., 2002), and 0.05–0.15 in Beagles (Steiner et al., 2001a).

For markers of intestinal absorptive function, the 6-h urinary recovery ratio of D-xylose to 3-O-methyl-D-glucose ranged from 0.60 to 0.90 in our healthy adult male laboratory Beagles, which was consistent with similar studies on a mixed group of Beagles (0.40–0.59) (Steiner et al., 2000, Steiner et al., 2001b), and in other breeds, including adult Miniature Poodles (0.52–0.65) (Weber et al., 2002), adult Standard Schnauzers (0.51–0.68) (Weber et al., 2002), adult Giant Schnauzers (0.54–0.62) (Weber et al., 2002), and adult Great Danes (0.56–0.62) (Weber et al., 2002).

As expected taking into account the breed, age, body size, sexual and health status, and possibly dietary effects on intestinal permeability in dogs, the ranges of the intestinal permeability markers (i.e. $^{51}$Cr-EDTA, lactulose and rhamnose) for healthy adult male Beagle dogs used in our studies were considerably different from, but in agreement with, the results of other investigations that have used heterogeneous groups of clinically healthy dogs. Conversely, findings from the intestinal function markers (i.e. D-xylose, 3-O-methyl-D-glucose) were in agreement with previous investigations suggesting that, in contrast to apparent morphological differences, the carrier-mediated mechanisms that allow intestinal absorption remain more similar between adult dogs of different breeds.

In our studies, the simultaneous administration of multiple markers was considered not to affect the recovery of each individual marker, as has previously been shown in dogs. Therefore, it is unlikely that any of the probe markers used in our examinations interfered with the permeation or absorption of the other molecules. (Steiner et al., 2000, Vaden et al., 2000, Steiner et al., 2002, Weber et al., 2002)

### 6.2 $^{51}$Cr-EDTA versus lactulose and sugar probes for intestinal permeability measurements (I)

Although $^{51}$Cr-EDTA and lactulose have markedly different physicochemical features, they have been regarded as equivalent intestinal permeability markers in humans and animals, including dogs. This is because of their similar molecular weight and cross-sectional diameter, and also because the rate of permeation through the intestinal wall revealed a high corresponding rate between the two markers after simultaneous administration in trials on humans and cats. When the intestinal permeation of $^{51}$Cr-EDTA and lactulose was analyzed after concurrent administration in humans and cats, both molecules showed a strong correlation when they were compared as single markers (humans: $r = 0.98$, $P = 0.001$; cats: $r = 0.85$, $P = 0.03$), or as ratios of both molecules against rhamnose (cats: $r = 0.97$, $P = 0.002$). (Maxton et al., 1986, Johnston et al., 2001)
Nevertheless, our data from study I (Table 6) suggest that the correlation between the percentage urinary recovery of $^{51}$Cr-EDTA and lactulose after their concurrent oral administration in Beagle dogs is not as prominent as previously observed in humans and cats when compared as single markers ($r = 0.64, P = 0.003$), or as ratios to rhamnose ($r = 0.50, P = 0.03$). The lower correlation between these two markers in our dogs may be explained by the possible partial degradation of the sugar probes, such as lactulose by intestinal enzymes (hydrolysis) or luminal bacteria (metabolization) of Beagles. However, a more likely reason for this disagreement is associated with intestinal breakdown of the sugar by intestinal resident bacteria, as it is well known that apparently healthy Beagles may be affected by small intestinal bacterial overgrowth, a dysbiotic condition that is consistent with intestinal degradation of saccharides such as lactulose. (Hall and Batt, 1991c, Batt et al., 1992, Hall and Batt, 1996, Riordan et al., 1997, Shen et al., 2009).

Our findings of a lower correlation between the recovery of $^{51}$Cr-EDTA and lactulose after their concurrent administration in Beagles suggest that caution should be exercised when intestinal permeability tests are carried out using saccharides, because dogs suspected of having intestinal dysbiosis (e.g. any dog with enteropathy) may yield false negative results. It is generally accepted that $^{51}$Cr-EDTA is a more sensitive intestinal permeability marker than the sugar probes because of its biological inertness and its resistance to intestinal bacterial degradation. Because the low correlation between $^{51}$Cr-EDTA and lactulose, it may be assumed that sugar probes may not be considered optimal markers to measure intestinal permeability in dogs, at least in Beagles.

No significant positive correlation was detected between the percentage urinary recovery of $^{51}$Cr-EDTA and other sugar markers such as rhamnose, D-xylose, 3-O-methyl-D-glucose, and sucrose, which is consistent with current knowledge on the recovery rates after intestinal permeation of such markers. Interestingly, the correlation between rhamnose, a marker reflecting non-mediated diffusion throughout the small intestine, and 3-O-methyl-D-glucose, a molecule that is very efficiently absorbed across the small intestine via a specific carrier-mediated transport system, was very strong ($r = 0.90, P = 0.000$). This may indicate that both markers are absorbed in a parallel manner by the intact small bowel mucosa of healthy Beagles. Support for this suggestion is added by the lower correlation ($r = 0.63, P = 0.004$) between rhamnose and D-xylose, whose intestinal absorption mechanism is similar to that of 3-O-methyl-D-glucose (i.e. via a specific carrier-mediated transport system) but limited to the jejunal mucosa, thus resulting in a lower absorption rate of D-xylose than of 3-O-methyl-D-glucose in the small intestine.

### 6.3 A single marker versus a combination of two for intestinal permeability measurements

It has generally been assumed that the ratio of two molecules provides a more reliable index of intestinal damage and dysfunction than single markers alone. However, the correlation rates observed in our studies in healthy Beagles between the percentage
recoveries of single markers (\(^{51}\text{Cr-EDTA}\) or lactulose) against their respective ratios to rhamnose (\(r = 0.73, P = 0.000\), and \(r = 0.80, P = 0.000\), respectively) suggest that the use of a single marker provides equivalent results to the use of a combination of two markers for assessing intestinal permeability in dogs. Furthermore, the analysis of one marker instead of two has practical and economic advantages.

6.4 The \(^{51}\text{Cr-EDTA}\) blood test in Beagles (II, III)

To the best of our knowledge, the \(^{51}\text{Cr-EDTA}\) intestinal permeability test in blood has never been attempted in human or veterinary medicine, and our aim was to develop it using laboratory Beagle dogs.

Findings from our studies indicate that it is possible to measure and calculate the percentage recovery of \(^{51}\text{Cr-EDTA}\) in the blood of Beagle dogs after oral ingestion of the probe molecule dissolved in water. The blood profile of the 6-h time course of \(^{51}\text{Cr-EDTA}\) showed equivalence with previous reports using \(^{51}\text{Cr-EDTA}\) as an intestinal permeability marker in the urine of healthy dogs. (Marks and Williams, 1998) Our results on the time course of \(^{51}\text{Cr-EDTA}\) in urine and blood over 6 hours provided further evidence that the absorption of \(^{51}\text{Cr-EDTA}\) across the canine intestine is more important during the first hours after its oral administration, and supports the proposed 6-h testing time as being more favorable than the 24-h test. The shorter testing time significantly increases the practicability of the test in dogs, increases the welfare of the subjects being housed in small metabolic cages for a more prolonged time, and allows the assessment of small intestinal permeability more specifically.

The statistical analysis of the results for the 43 observations in study II revealed a strong correlation between the recovery of \(^{51}\text{Cr-EDTA}\) in urine and blood. Recovery rates determined for single blood samples correlated well with the cumulative 6-h urine recovery, especially when a blood sample was taken at 4 hours (\(r = 0.89\)) (Table 10). However, the correspondence between urine and blood was significantly better when results for individual blood samples were combined. The correlation coefficient between results in urine and multiple blood samples was highest when four blood samples were summed (\(r = 0.97\)), and remained excellent when only two blood samples taken at 3 and 5 h (\(r = 0.95\)) or at 3 and 4 h (\(r = 0.94\)) were summed (Table 10). It was expected that the sum of percentages in sera would correlate better with urine than single blood samples, because the latter is measured at one point in time whereas the former more closely resembles the cumulative excretion of \(^{51}\text{Cr-EDTA}\) in urine. Additionally, the stability of these findings was confirmed by the results on the correlations between urine and sera from the 6 dogs that underwent intestinal permeability testing thrice.

The levels of \(^{51}\text{Cr-EDTA}\) in both serum and plasma after oral ingestion of the probe molecule in dogs were strongly correlated (Fig. 8). This demonstrates that the \(^{51}\text{Cr-EDTA}\) intestinal permeability blood test in dogs can be equally performed on either serum or plasma samples, and supports the use of plasma when a small amount of blood
is collected from the patient. These correlations were further analyzed using the Bland–Altman test, which revealed no differences in $^{51}$Cr-EDTA levels between serum and plasma samples. The regression line of differences versus means and 95% confidence intervals was additionally included, illustrating a linear regression between the 2-sample tests. Therefore, the intestinal permeability test measured in blood showed no differences in $^{51}$Cr-EDTA levels of serum or plasma, evidencing that there are no differences in the two methods used for assessing intestinal permeability in dogs.

6.5 Iohexol as a potential intestinal permeability marker in dogs (IV)

In study IV, a strong correlation including a clear linear association was detected between $^{51}$Cr-EDTA and iohexol during the 6-h time course in serum after concurrent oral administration of both markers, suggesting that they follow a similar mucosal permeability pathway across the intestinal wall. The percentage serum recovery values of $^{51}$Cr-EDTA were significantly higher than iohexol at each time point, which may be explained by the lower molecular weight (359 Da) and the smaller cross-sectional diameter of $^{51}$Cr-EDTA (10.5 Å) compared to iohexol (821 Da and 12 Å, respectively). This led to a higher absorption rate across the gut mucosa and thus higher serum levels of $^{51}$Cr-EDTA compared to iohexol (Hollander et al., 1988, Andersen et al., 1996, Andersen et al., 2001).

These findings are consistent with previous reports comparing $^{51}$Cr-EDTA and iohexol in animal models and humans, and because $^{51}$Cr-EDTA and iohexol share similar physicochemical properties and follow a similar mucosal permeability pathway across the intestinal wall, it would be expected that the results obtained with their use in health and disease would be similar.

Nevertheless, further studies are warranted using iohexol as an intestinal permeability blood marker in dogs under clinical conditions altering the gut mucosa to confirm the validity of the iohexol intestinal permeability blood test as a diagnostic tool for use in research and routine clinical practice.

Although additional investigations will need to test whether iohexol truly detects gut injury and qualifies as a permeability marker, these results provide promise for further testing using clinically affected dogs.

6.6 Preclinical evaluation of iohexol as intestinal permeability marker using a well-characterized experimental intestinal disease rat model (V)

Laboratory Sprague-Dawley rats were used to replace an experimental disease model potentially induced in dogs. This was planned and executed in accordance with the guiding principles of replacement of laboratory animal use, since the features and symptomatology of the intestinal disease condition could be reproduced in the rat, an

As previously demonstrated in laboratory rats, our findings from study V provided further evidence that intestinal permeability may be assessed in Sprague-Dawley rats by measuring the cumulative urinary excretion of an orally-administered dose of iohexol, using a different test protocol aimed at improving the welfare of the test subjects. In our studies, increased intestinal permeability was reflected in a higher excretion of iohexol in urine due to a higher permeation rate of the probe across the damaged intestinal mucosa of animals with enteric abnormalities. In our laboratory Sprague-Dawley rats, the median 24-h urinary recovery after the oral administration of iohexol was 0.54% in healthy individuals, and 11.42% in rats with a well-characterized experimentally-induced inflammatory bowel disease, indicating significantly higher excretion of the contrast medium in rats with enteropathy. These findings were in agreement with the values reported by other research groups using iohexol in rats with experimental enteropathies, providing further support for the valid use of iohexol in detecting intestinal alterations in a rat model of inflammatory bowel disease. (Stordahl, 1988a, b, Stordahl and Laerum, 1988a, b, Laerum et al., 1990, Solheim et al., 1991, Andersen et al., 1992, Andersen et al., 2001)
7. CONCLUSIONS

1. Normative data were provided on the percentage urinary recovery after a 6-h oral administration of the gastrointestinal permeability and function markers $^{51}$Cr-EDTA, lactulose, rhamnose, D-xylose, 3-O-methyl-D-glucose, and sucrose to healthy adult male laboratory Beagle dogs that were used and cared for under controlled laboratory conditions. This is the most commonly used breed in animal research and one of the most popular companion breeds worldwide (in the top 3 list of the American Kennel Club for 2011). These data add to current knowledge on the normal gastrointestinal mucosal integrity and absorptive capacity for this dog breed, type, and environment.

2. The correlation between the percentage urinary recovery of intestinal permeability markers $^{51}$Cr-EDTA and lactulose in healthy adult Beagles was not as prominent as previously reported for humans and cats. The reason for this discrepancy was not specifically investigated, but it is likely to be associated with the degradation of the sugar probe by intestinal resident bacteria. Further studies may be needed for full clarification, but caution should meanwhile be warranted when using and interpreting gastrointestinal permeability and function test results obtained using lactulose and other sugar probes. This is particularly the case in dogs suspected of having intestinal dysbiosis, such as Beagles or clinical patients affected with gastrointestinal disease.

3. It has been claimed that intestinal permeability results obtained by only using a single marker may be affected by non-mucosal factors during intestinal permeation. However, the percentage urinary recovery of $^{51}$Cr-EDTA and lactulose used as single intestinal permeability markers, and the ratio of these same molecules against rhamnose ($^{51}$Cr-EDTA/rhamnose and lactulose/rhamnose, respectively), provided a strong and very significant correlation. This suggests that the use of a single marker may be sufficient and more practical for assessing intestinal permeability in healthy dogs, and the same is probably also true in dogs affected with an intestinal disorder.

4. The $^{51}$Cr-EDTA permeability test in blood addresses the problems associated with urine collection in dogs, and obviates the laborious sample preparation that is required for the sugar probes. The blood approach based on the collection of at least two blood specimens gives results that are comparable with the 6-h cumulative urine test, and can thus provide a means of assessing intestinal permeability in dogs. Multiple blood sample collection (i.e. 3–5 samples) provides slightly better correlation results with the urine-based test than a single blood sample, and this would be therefore recommended when the test’s sensitivity is to be increased. Nevertheless, further studies are needed on dogs with intestinal disease in order
Conclusions

to define a cut-off value that would enable normal and abnormal individuals to be discriminated with more precision.

5. The use of plasma or whole blood during the $^{51}$Cr-EDTA intestinal permeability blood test may be preferable to the use of serum in certain patients in which a lower volume of blood sample is anticipated, such as in juvenile animals or miniature dog breed patients. It was concluded that the choice between serum or plasma when performing the $^{51}$Cr-EDTA intestinal permeability blood test in dogs is not relevant and is only one of convenience.

6. A clear relationship between serum levels of $^{51}$Cr-EDTA and iohexol was recorded when they were simultaneously administered, suggesting that both molecules behave similarly as blood markers in the assessment of intestinal permeability in dogs.

7. The intestinal permeability test using iohexol in a laboratory rat model with a well-characterized experimentally-induced intestinal disease was able to clearly discriminate between healthy animals and rats with intestinal mucosal damage.

8. Additional studies using iohexol as an intestinal permeability blood marker in dogs under clinical conditions, in which the gut mucosa is altered, are required to confirm the validity of the iohexol intestinal permeability blood test in dogs for future clinical and scientific use.
8. ACKNOWLEDGEMENTS

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