INVESTIGATION OF DOG URINE STABILITY FOR USE IN METABOLOMICS, STABILITY OF ADVANCED GLYCATION END PRODUCTS, & RECOMMENDATIONS FOR SAMPLE COLLECTION, HANDLING & STORAGE

by

NICOLE RENEE CAMMACK

(Under the Direction of Joseph W. Bartges)

ABSTRACT

Metabolomics has been integrated into human nutrition research, enabling scientists to evaluate how dietary components influence health. These advancements have occurred because of standardization of biofluid handling, metabolite stability assessment, and database development. In contrast, canine metabolomics remains underutilized, particularly in the context of evaluating diets and their relationship to chronic disease. Despite dogs sharing many of the same diet-related conditions as humans, including obesity, diabetes mellitus, and cognitive decline, several challenges limit their use as translational models.

This dissertation explores veterinary metabolomics and related research in dogs. First, a systematic review identified substantial inconsistencies in canine urine sample collection, handling, and reporting, restricting reproducibility and data comparability. To address this, we evaluated short-term metabolic stability in canine urine stored under refrigeration and room temperature using untargeted ^{1}H NMR. A second study evaluated the stability of two diet-associated advanced glycation end products (AGEs), Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL) in dog urine stored at ambient temperature for up to 168 hours.

Minimal degradation was observed, supporting the feasibility of urinary AGE research in dogs under variable storage timelines. Lastly, we present recommendations for dog urine sample collection, handing, and storage to improve the quality of dog metabolomics research. This work provides recommendations for urine collection and storage for canine metabolomics and AGE research.

INDEX WORDS: dog metabolomics, urine metabolomics, Maillard reaction, advanced glycation end products, canine metabolome

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DEDICATION

To Taser, Dasso, and Beau,

This work would not exist without my incredible dogs and the wonderful life they have given me. Taser, my beloved late Pit Bull, taught me to never give up and never take no for an answer. I will be forever grateful for his unwavering companionship, loyalty, and zest for life. He introduced me to pet nutrition, alongside his brother Beau and late brother Dasso, inspired my business and helped improve the lives of many pets and people.

Dasso, my brave pittie who came into my life as an adult rescue, taught me the value of patience and how special a bond with a dog so profoundly let down by humans could be. Your kind nature and ability to see the good despite your past remain with me always.

Beau, my faithful Dachshund, my cuddle buddy and supervisor, has been by my side for 16 years. I couldn't imagine a better study or writing companion. You've spent more hours studying and working on my lap or next to me in my chair for (our) degrees. This PhD is just as much yours as it is mine. Your companionship is a constant source of motivation and joy.

After Dasso passed, Tase and Beau moved with me from Connecticut to Georgia. While losing Tase was the greatest loss I've ever endured, but your story will forever carry me. Tase, your boundless energy and unbreakable spirit taught me the true meaning of resilience. Your love was a constant light in my life, guiding me through every challenge.

To each of you, thank you for the love, inspiration, and unwavering loyalty you have given me. You are the reason behind my passion for pet health and nutrition, the driving force of my business, the inspiration for my PhD and future research.

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CHAPTER 1

INTRODUCTION

Metabolomics is encompassed within broader 'omics' technologies, including genomics, transcriptomics, and proteomics, and provides insights into the dynamic physiological states of organisms. It advances understanding and knowledge of health and disease, improving disease management and therapeutic development across species. Metabolomics has advanced precision medicine by identifying metabolite profiles linked to disrupted metabolic pathways, dietary patterns, and genetic conditions in humans (Abdul-Hamid et al., 2013; Clish, 2015; Cossu et al., 2023; Jones et al., 2012; Wishart, 2019). However, similar advancements have not been realized in canine metabolomics despite dogs sharing more than 360 diseases with humans, along with similar metabolic traits, environmental exposures, and possibly consumption of ultra processed pet foods (Parker & Ostrander, 2005; Patterson, 2000; van Rooijen et al., 2014; Wayne & Ostrander, 2007). Dogs shorter lifespans and controlled environments make them strong translational models for longitudinal research on aging, nutrition, and chronic disease (Graciela et al., 2020; Wishart, 2019).

In humans, metabolomics is used not only for newborn screening but also to detect adult-onset metabolic disorders, kidney disease, and certain cancers that previously have been missed or misdiagnosed (Cossu et al., 2023). Such advances are driven by improvements in standardized methods, technology and bioinformatics that allow detection of low-abundance metabolites (Clish, 2015). Nevertheless, similar integration in veterinary practice is lacking despite shared genome and disease parallels between humans and dogs (Kirkness et al., 2003).

Metabolomics enables identification of disrupted metabolic pathways, dietary effects, and genetic conditions (Wishart, 2019). In dogs, urine metabolomics remains underutilized but has high potential, especially for investigating shared diseases like diabetes mellitus, cancer, and obesity. Metabolism includes all biochemical processes within and between cells, not just digestion (Sánchez Lopez de Nava, 2024). Therefore, detection and quantification of relevant metabolites and patterns supports biomarker discovery and mechanistic insight into health and disease. Current gaps in veterinary medicine include a lack of standardized urine handling protocols, limited metabolite stability data, and incomplete consideration of processing effects of diet on health and disease in dogs.

High-thermal food processing of food generates various byproducts, including advanced glycation end products (AGEs), that are abundant in ultra-processed foods. In human nutrition, ultra-processed foods are recognized to be hyper-palatable, have prolonged shelf lives, and may be consumed on-the-go (Monteiro et al., 2018). These foods are not simply modified whole foods, but are rather entirely or nearly entirely comprised of industrial formulations containing fractionated ingredients and additives without any recognizable whole foods (Monteiro et al., 2018). Ultra-processed foods may include fiber, certain nutrients, or reduced fat that imply perceived healthfulness despite health risk (Monteiro et al., 2010). In humans, ultra-processed food and AGEs are associated with multiple conditions, poor prognosis, and all-cause mortality (Chaudhuri et al., 2018; Poulsen et al., 2013), although some experts debase these associations citing lack of detailed cause and effect mechanisms (Hellwig et al., 2024). Biological influences of AGEs are not limited to dietary intake. The identification of HbA1c in diabetic patients revealed that the Maillard Reaction, previously associated with food processing, also occurs

endogenously and contributes to the formation of AGEs within the body (Rahbar et al., 1969), and since driven interest in the role of dietary and *in vivo* AGEs in health across species.

The NOVA classification system categorizes foods based on extent and purpose of processing (Monteiro et al., 2010). Research utilizing NOVA has associated numerous health risks with consumption of ultra-processed foods (Monteiro et al., 2018). Pet food classification systems are non-existent compared to human frameworks like NOVA, despite processing methods influence nutritional quality (Raditic, 2021; van Rooijen, 2015), and possibly health outcomes. In addition, studies have shown dogs consuming commercial diets ingest 122-fold more AGEs on a metabolic body weight basis than humans consuming a typical Western diet (van Rooijen et al., 2014).

Stability data for food processing by products like AGEs and other metabolites in dog urine are limited despite growing interest, increased publications, and rise of biobanking initiatives. These present barriers to integrating AGE research into investigations involving food processing, nutrition, and metabolomics (Lehmann, 2021). Without standardized protocols for sample collection, handling, and storage, reproducibility and accuracy in identifying both known and novel biomarkers is compromised (Abdul-Hamid et al., 2015; Raúl González-Domínguez, 2020; Wishart, 2019). This is especially relevant in studies aiming to understand how diet influences metabolic health, where even minor pre-analytical inconsistencies can obscure biological information. AGEs, as dietary components and endogenous metabolites, are influenced by food processing and health, making them valuable biomarker for investigating nutrition and metabolic profiling. Advancing metabolomics in this context requires consensus on methodological standards to support meaningful cross-study comparisons and to enable

development of classification systems that reflect both nutrient composition and processing intensity.

This dissertation contributes a standardized protocol for canine urine metabolomics to improving consistency and reliability in sample handling, particularly for biomarker discovery, including AGEs, and disease research with translational relevance. By evaluating the short-term stability of AGEs in urine, we address a methodological gap and supports broader metabolomics applications in both veterinary and comparative contexts. Together, these methodological advances lay the groundwork for future studies exploring the role of ultra-processed diets, dietary AGEs, and chronic disease across species, enhancing diagnostic, therapeutic, and nutritional strategies in both veterinary and human medicine.

Research Objectives

The objectives of this work were to:

1. Evaluate the stability of AGEs, specifically carboxyethyl lysine (CEL) and carboxymethyl lysine (CML), in dog urine up to 7 days at room temperature.

2. Evaluate sample stability of dog urine subjected to different time and temperature conditions for use in metabolomics studies.

3. Establish a set of sample collection, handling and storage recommendations based on our stability investigations and human recommendations, adapted for use in dog urine metabolomics and CEL and CML studies.

CHAPTER 2

LITERATURE REVIEW

Metabolomics has advanced understanding of health and disease by enabling precise systems-level insights into physiological and pathological states. While its application has accelerated therapeutic discovery and disease management in human medicine, use in veterinary science, particularly dogs, remains in its infancy, limiting transitional potential across species. Compared to human research, canine metabolomics lacks methodological standardization resulting in analytical challenges and limitations in the field. Further, these gaps restrict the ability to draw meaningful conclusions about long-term dietary exposures in dogs, including their impact on metabolic health, nutrient bioavailability, and the accumulation of dietary byproducts. Limitations in study design, sample handling, and reporting standards further constrain efforts to assess how diet influences chronic disease risk, biochemical pathways, and inflammatory processes over time. Understanding relationships between diet, metabolomic profiles, and health outcomes requires consideration of multiple factors. This includes pathways of advanced glycation end products (AGEs) and other Maillard reaction product (MRP) formation and their roles chronic disease. AGE accumulation has been well characterized in human models and is likely relevant to companion animals. These biochemical mechanisms, along with processing byproducts and regulatory blind spots, provide the foundation for evaluating the limitations of current nutritional standards and the need for a framework that accounts for processing intensity, metabolic health, and long-term outcomes.

2.1 Human Metabolomics

Metabolomics involves detection and analysis of a variety of metabolites including nutrients, drugs, and signaling mediators in biological samples (Liu & Locasale, 2017; Rinschen et al., 2019; Schmidt et al., 2021). It has identified biomarkers that contribute to tumorigenesis, inflammation, and metabolic characteristics of chronic diseases. Cancers exhibit unique metabolic signatures used to develop diagnostic testing, and therapeutic targeting and response to treatment, notably prostatic, hepatic, pancreatic, and breast cancers (Budhu et al., 2014; Schmidt et al., 2021). For example, increased concentrations of fumarate and succinate from the citric acid cycle occurring in certain tumor types inhibit cellular processes by modifying transcription and promoting uncontrolled growth, thus, supporting the role of these metabolites as oncometabolites (Richter et al., 2014; Yang et al., 2012).

Metabolomic alterations including amino acid, carbohydrate, vitamin cofactor, energy, lipid, nucleotide, peptide, and xenobiotic metabolism occurs in obesity (Cirulli et al., 2019), highlighting disease complexity and unique metabolic profiles among individuals. Similarly, metabolic analysis has identified multiple pathways and metabolite patterns predictive of osteoarthritis (OA) risk and disease progression (Zhai, 2021). In chronic kidney disease (Schlosser et al.), metabolomics uncovered potential biomarkers for early renal injury detection and risk assessment while increasing understanding of disease pathophysiology and severity (Lousa et al., 2021).

2.1.2 Metabolomics Databases

The Human Metabolome Database (HMDB, https://www.hmdb.ca), a free, internet-accessible tool, provides detailed chemical, clinical, molecular biological, and biochemical data for advancing metabolomics, clinical chemistry, biomarker discovery, and education (Wishart et al.,

2009). The HMDB currently contains 20,610 metabolites including 3,376 quantified compounds (Bouatra et al., 2013). Of these, 3,564 metabolites have been detected in urine, with 1,844 quantified, with additional metabolites cataloged across other biological samples including cerebrospinal fluid, blood, and saliva (Table 2.1).

Table 2.1. Human Metabolome Database. Summary of the number of metabolites identified and quantified in various human biological samples. Date Accessed: May 27, 2024.

Human Metabolome Database			
Biological Sample Type	**Detected & Quantified**	**Detected (unquantified)**	**Total**
Blood	3,126	12,003	15,129
Urine	1,844	1,720	3,564
Saliva	886	351	1,237
Cerebral Spinal Fluid	448	2	450
Feces	1,812	4,977	6,789
Sweat	79	13	92
Breast Milk	102	20	122
Bile	18	0	18
Amniotic Fluid	18	0	18
Other	18	0	18

The Metabolomics Workbench (https://www.metabolomicsworkbench.org), sponsored by the Common Fund of the National Institutes of Health (NIH) and hosted by the University of California at San Diego, offers another publicly accessible database (Workbench, 2024). As of February 2024, the platform provides access to over 169,000 entries on metabolite structures and annotations in various biofluids. The Biological Magnetic Resonance Data Bank (BMRB, https://bmrb.io) provides similar resources but specific to Nuclear Magnetic Resonance (NMR)

metabolomics. Current metabolomic platforms have enabled detection and profiling of metabolite subsets in various biospecimens seen in these databases; however, substantial portions of the human metabolome remain uncharacterized (Jones, 2018), and is termed the 'dark metabolome' (Dias et al., 2016).

2.1.3 Urine Metabolomics Sample Collection & Handling

Accurate detection and quantification of metabolites in biospecimens relies upon validated and consistent sample collection and handling alongside optimized study design (Wishart, 2019). Time delays, temperature fluctuation, and contamination prior to storage and data acquisition result in metabolite instability in biosamples (Dorota et al., 2022; Hai et al., 2020; Rainer Lehmann, 2020; Raúl et al., 2020). While hematological analyses are more common, urine has unrealized potential in metabolomics due to non-invasive collection methods and metabolite profile (Bouatra et al., 2013).

Metabolomic study design requires careful consideration for sample collection. Human studies have demonstrated importance of standardized sample collection protocols in metabolomics studies to reduce pre-analytical variability and improve reproducibility across datasets. Urine collection methods (e.g. such as free-catch, cystocentesis, or catheterization) should remain consistent within a study to minimize methodological variability. Collection timing should also be standardized, with first-morning voids commonly recommended due to diurnal shifts in urine composition (Dallmann et al., 2012). Feeding status before and during sampling should be controlled, when possible, as nutrient intake significantly impacts metabolite profiles. Likewise, physical activity and environmental exposures have been shown to influence the metabolome and should either be standardized or carefully recorded to reduce confounding variables (Emwas et al., 2015; Jianqiang Wu, 2015). Despite these recognized influences,

deviations from standardized collection and subject conditions remain common in veterinary metabolomics and contribute to challenges in data interpretation and cross-study comparison.

In addition to collection methods, appropriate handling and container selection are essential to preserve sample integrity. Inert containers, (e.g. polypropylene), are widely recommended due to low chemical reactivity and minimal interaction with urine components (Raúl et al., 2020; Yao et al., 2016). However, consistency in both the type and manufacturer of containers is important, as variability may lead to differential chemical leaching or adsorption, introducing unwanted analytical noise (Raúl et al., 2020; Yao et al., 2016). For aliquoting, polypropylene cryovials are also commonly used, but their permeability to CO_2, especially when frozen on dry ice, may alter sample pH (Rist et al., 2013; Yao et al., 2016). These considerations, while standard in human metabolomics, are frequently underreported in canine studies, reinforcing the need for transparent and consistent protocols to support comparability and data quality.

2.1.4 Urine Metabolomics: Storage & Stability Considerations

Storage at 4 °C: Metabolite Stability up to 24 Hours

Urine samples can be stored at 4 °C for short durations, particularly when immediate freezing at -80 °C is not feasible. Studies have demonstrated urine metabolite profiles remain stable at 4° C at least 24 hours, if samples have been collected on ice, and gently centrifuged (1,000-3,000 RCF) to remove cells and debris without inducing cell lysis (Aurelie et al., 2015; Budde et al., 2016; Gika et al., 2008; Patrizia et al., 2011).

2.1.4.2 Storage at -20 °C & -80 °C: Metabolite Stability up to 6 months

Published data has demonstrated stability of urine metabolites when stored at -20°C for up to 6 months, with minimal impact on the metabolite profile, especially if the sample is subjected to

mild centrifugation (1,000-3,000 RCF) before freezing (Gika et al., 2008; Laparre et al., 2017; Lauridsen et al., 2007; Stevens et al., 2019). Other studies concluded urine stored at -20 °C or -80 °C for 1-month did not measurably affect metabolomic data (Gika et al., 2008; Stevens et al., 2019).

2.1.4.3 Long Term Storage at -80 °C: Metabolite Stability 12 months to 5 years

Long term storage of urine at -80 °C for 12 months is acceptable in metabolomics biobanking practice, although few studies beyond 6 months at -80 °C are available. Ghini et al. (2019) demonstrated that when mild centrifugation (1,000-3,000 RCF) and filtration (0.2 μm filtered pipette tip) are utilized to remove cellular and bacterial content, urine samples stored at -80 °C remain stable up to five years, with negligible changes (Ghini et al., 2019). This supports that deep-frozen, well-prepared urine samples are suitable for delayed analysis. In contrast, unprocessed urine exhibited pH drift, signal shifts, and metabolite degradation over time, demonstrating importance of standardized pre-analytical protocols to preserve reproducibility and biological interpretability of data from biobanked samples (Ghini et al., 2019).

2.1.4.4 Alternative Freezing & Storage Methods

Ideal urine freezing and storage via liquid nitrogen, or nitrogen vapor is likely ideal compared to previously mentioned methods. This is because freezing at -130 °C allows for bypass of centrifugation and filtering steps altogether, as it prevents cell lysis and associated challenges (Patrizia et al., 2011). Factors limiting widespread use of this likely include cost, access and logistical considerations.

2.1.4.5 Current Sample Handling & Storage Recommendations

Current metabolomics recommendations emphasize consistency in biobanking practices to preserve sample integrity, regardless of method. Researchers should take care not to assume

indefinite stability, regardless of storage method, since it is possible extremely labile metabolites could slowly degrade even at -80 °C, and container effects (e.g. adsorption to tube material/contaminants, etc.) may occur over multi-year spans.

2.1.5 Metabolomics Data Acquisition

Targeted metabolomics detects and quantifies specific, predefined metabolites providing quantitative or semi-quantitative results suitable for hypothesis-driven studies investigating specific pathways or disease states. Untargeted analyses provide broad metabolic profiles of detectible known and unknown metabolites within biosamples making it ideal for hypothesis-generation and biomarker discovery. Use of targeted and untargeted methods, especially when used together, can provide detailed information about an organism's metabolomic status where untargeted metabolomics facilitate discovery of novel biomarkers and targeted metabolomics quantify and validate them.

Metabolomics data acquisition platforms commonly include mass spectrometry (MS)-based and NMR spectroscopy-based techniques. MS-based methods predominant due to versatility and the ability to detect tens of thousands of features in a sample with reference spectra contained in numerous databases (Edison et al., 2020). It is a high-resolution technique enabling broad-spectrum metabolite detection providing qualitative and quantitative data across diverse biosamples. MS is often enhanced by complementary techniques like gas chromatography (GC-MS) or liquid chromatography (LC-MS) and utilized for targeted analyses and to a lesser extent non-targeted studies (A. H. Emwas et al., 2019). A limitation is that MS is often destructive, usually preventing reanalysis of a sample (A.-H. Emwas et al., 2019; A. H. Emwas et al., 2019).

NMR spectroscopy is used to discover biomarkers and investigate pathways regulating cellular and physiological processes (Edison et al., 2020; A.-H. Emwas et al., 2019). One-dimensional and two-dimensional techniques enable identification and quantification of metabolites with minimal sample preparation and without destroying the sample (Dozio et al., 2023; Edison et al., 2020; Gouveia et al., 2021; Hooshiar et al., 2022; Markley et al., 2016). NMR is highly reproducible, suited for untargeted and targeted analyses with notable strengths in untargeted analyses due to its unbiased detection of a broad range of polar metabolites, including those poorly captured by LC-MS; making it ideal for large-scale, high-throughput studies (A. H. Emwas et al., 2019). Additional advantages include utility in noninvasive *in vivo* studies, real-time or discrete timepoint analysis of biosamples or cell culture media and identification of unknown compounds (Edison et al., 2020; A. H. Emwas et al., 2019). Limitations of NMR include overlapping peaks from metabolite profiles often posing challenges in data acquisition and analysis; however, advanced computational analytical methods alleviate some of these (A. H. Emwas et al., 2019; Y. Wu et al., 2024). NMR data acquisition and sample composition is influenced by factors such as temperature, pH, and instrument settings, which also alters metabolite stability and cause chemical shift variability (JC et al., 2004). Standardized protocols are therefore required to minimize experimental variation and improve reproducibility across samples and studies (Wishart, 2019).

Given the heterogeneity of metabolites and metabolite classes, including AGEs, no single analytical platform can capture all metabolites within a biological sample demonstrating that MS, NMR and other platforms are complementary rather than competing (Blaženović et al., 2019; Edison et al., 2020). Optimal analytical techniques and strategies are best determined by research objectives, sample type, metabolites, and specific metabolite classes of interest.

2.1.6 Considerations for Storage and Reanalysis of Prepared Urine Samples for NMR

Post-Analytical Sample Freezing

In NMR metabolomics, urine samples are typically prepared with phosphate buffer (~0.1 M KH_2PO_4) and sodium azide to stabilize pH and inhibit microbial growth during data acquisition (Anthony C. Dona, 2014). These buffered samples are often assumed to remain stable when frozen at −20 °C for future analysis, or reanalysis after initial data acquisition. Short-term reanalysis appears valid as a study reported negligible changes in ^{1}H NMR profiles of rat urine after 5-8 freeze-thaw cycles or storage at −20 °C for up to 24 months (Schreier et al., 2013). However, their study was limited to 1D NMR and did not assess 2D spectral quality or potential crystallization effects. Thus, while -20 °C storage may preserve 1D NMR utility for brief periods, long-term stability, particularly for buffered samples, requires further validation, especially for techniques more sensitive to sample heterogeneity such as 2D NMR.

2.1.6.2 Crystallization & Precipitate Formation in Buffered Post-Analytical Samples

Crystallization and precipitate formation are often overlooked challenges in metabolomics when buffered urine samples are stored frozen such as -20 °C. Urine contains high concentrations of ions such as calcium, oxalate, phosphate, and urea, which may exceed solubility limits upon freezing. Even in centrifuged samples, visible precipitates like calcium oxalate dihydrate and amorphous calcium salts have been observed after overnight storage at -20 °C and can persist after thawing (Saetun et al., 2009). These precipitates may trap analytes, alter sample composition, or interfere with downstream analysis. Additionally, phosphate buffers used to stabilize pH can crystallize during freezing. For example, sodium phosphate buffers may form hydrates, shifting the monobasic-to-dibasic ratio and causing pH drift (Gomez, 1995).

These changes have direct consequences for NMR metabolomics. High-resolution NMR assumes a homogenous solution and undissolved particulates introduce magnetic field inhomogeneities that impair shimming and degrade spectral resolution. While 1D ^{1}H NMR may be regained because crystals settle and the supernatant remains analyzable, 2D NMR is more susceptible to distortion due to longer acquisition times and greater sensitivity to subtle matrix changes. Co-precipitation of analytes and altered pH further compromise spectral quality and quantitation. Together, these physical and chemical instabilities in the sample itself reduce reproducibility and data integrity, particularly in sensitive experiments. These findings demonstrate importance of rigorous pre-analytical processing and storage protocols, especially when storing buffered urine samples for later reanalysis such as with 2D NMR.

2.2 Dog Metabolomics

Canine biosamples have been used to investigate urinary tract disorders, hepatic dysfunction, metabolic irregularities, inborn errors of metabolism, infectious disease, aging, diet, and neoplasm (Ferlizza et al., 2020; Lawrence et al., 2019; O'Kell et al., 2019; Sewell et al., 2007; Söder et al., 2017; Taibo et al., 2023; Tsamouri et al., 2022). The scope of these studies remains narrow and small compared to human studies.

There are distinctive differences in urine metabolomic signatures between juvenile and adult dogs with profiles correlating to development and physiological status (Taibo et al., 2023). Juvenile dogs exhibited upregulation of the pentose phosphate pathway and protein digestion and absorption, which were downregulated in adult dogs. This initial age-related metabolic characterization establishes a framework for investigating age-related diseases, biomarker discovery, and developing precision medicine approaches.

Postprandial differences in the urinary metabolome were found between overweight and healthy-weight dogs (Söder et al., 2017). Overweight dogs exhibited lower postprandial urinary taurine concentrations compared to controls suggesting correlation between low taurine and increased adiposity. This finding suggests increased risk for conditions such as dilated cardiomyopathy (DCM) that has been linked to taurine deficiency (Sanderson et al., 2001). Beyond cardiac health, decreased taurine alters bile acid conjugation, detoxification, nervous system function, thrombus formation, reproduction, cellular homeostasis, membrane stabilization, and antioxidative status (Bae et al., 2022; Jo et al., 2019; Kendler, 1989; Schaffer et al., 2014; Warskulat et al., 2004; Wu & Prentice, 2010). The complex nature of these interactions highlights the need for further research into relationships between obesity and comorbidities, and related nutritional factors.

Urine metabolomic analysis has the potential to detect dietary patterns and identify nutritional biomarkers linked to specific foods or diets (Clarke et al., 2023; Su et al., 2023; Willis et al., 2020). Distinctive metabolite signatures and associated pathway alterations are documented in several canine diseases including urothelial carcinoma, chronic hepatic disease, mammary cancer, and atopy (Lawrence et al., 2019; Lee et al., 2022; Moore et al., 2020; Tsamouri et al., 2022; J. Zhang et al., 2012). Collectively, these findings demonstrate the capability of metabolomics to discover altered metabolic characteristics within a wide range of pathological conditions in dogs as in humans.

2.2.2 Dog Urine Metabolomics, Sample Collection, Handling, & Storage

Human urinary metabolomic studies have reproducible and reliable results due to established recommendations and protocols for biosample collection, handling, storage, and sample preparation procedures (Abdul-Hamid et al., 2015; Abdul-Hamid Emwas, 2016; Dinges et al.,

2019; Lauridsen et al., 2007; Rainer Lehmann, 2020; Raúl et al., 2020). These standardizations resulted from stability investigations examining pre-analytical conditions' impact on integrity of metabolites in urine; however, veterinary medicine lacks comparable standardizations for canine urinary metabolomics investigations and dog urine metabolomics studies often deviate from established human protocols. Lack of standardized pre-analytical steps often seen in these studies introduce variability and complicate studies investigating disease characterization, biomarker identification, and therapeutic development introduces variation that compromise results (Braisted et al., 2024). Profiling studies, especially those seeking to identify target biomarkers, need to be well documented and repeatable. Thus. sample integrity should be prioritized to achieve repeatability, and collection, handling, storage, and preparation methods should be reported in detail (Wishart, 2019). Therefore, precise dog urine collection, handling, and storage recommendations are required to ensure quality of results and method reproducibility.

2.3 Comparative Research Challenges & Opportunities

The domestic dog, *Canis familiaris*, has been reported to develop over 450 diseases, sharing approximately 360 of those with human diseases (Parker & Ostrander, 2005; Patterson, 2000; Wayne & Ostrander, 2007). As such, dogs are relevant comparative models for human biomedical research utilizing metabolomics, due to shared environments, lifestyle patterns, dietary intake, and susceptibility to develop similar conditions, including cancer, degenerative joint disease, Alzheimer's Disease/canine cognitive dysfunction, chronic kidney disease, obesity, metabolic disorders, and diabetes mellitus (Abdelsattar et al., 2021; Abdul-Hamid et al., 2015; Abdul-Hamid et al., 2013; Alexander et al., 2011; Budhu et al., 2014; Cirulli et al., 2019; Deidda et al., 2015; Forster et al., 2018; Huo et al., 2020; Lee et al., 2022; O'Kell et al., 2019; Qu et al., 2022; Söder et al., 2017). To date, over 45 meta-analyses associate ultra-processed food or AGEs

with these diseases as well as poor prognosis, and all-cause mortality in humans. Despite established associations, substantial gaps remain particularly in dogs who consume predominantly processed commercial diets containing up to 122-fold higher AGEs, specifically HMF, than human diets on a metabolic body weight basis (van Rooijen et al., 2014). Dogs can serve as comparative models for investigating AGEs and similar conditions.

Human clinical research faces well-recognized limitations in characterizing diseases for early detection and management (Li et al., 2024; Ulmer et al., 2021). One notable challenge includes complexity in controlling infinite variables influencing health and disease, such as diet, environmental exposure, genetic factors, and physical activity. Another is longitudinal human studies present substantial logistical, financial, and ethical challenges related to extended timeframes necessary for completion. Dogs are useful models because of shorter lifetimes and greater control of living conditions facilitating lifetime studies in realistic timeframes.
While dogs are valuable biomedical research models, challenges remain in applying metabolomics methodologies. Absence of species-specific databases and established protocols limits the scope and robustness of metabolomic profiling across various breeds, ages, populations, cancers, metabolic disorders, and more. Nevertheless, despite differences, biological similarities between species offer opportunities to address research gaps.

2.3.2 Metabolomics for Studying Food & Intake Biomarkers

Metabolomics has uncovered metabolites indicative of nutritional intake in rodents, humans, and dogs (Clarke et al., 2023; González-Peña & Brennan, 2019; Hall et al., 2011; Huybrechts et al., 2022; Kortesniemi et al., 2023; Mohtashamian et al., 2022; Su et al., 2023; Willis et al., 2020; Zhang et al., 2013). From a nutritional and food processing standpoint, metabolomics is useful for studying the physiological impact of food processing derived compounds on health

outcomes. Such compounds include AGEs, acrylamides, heterocyclic amines, biogenic amines, and pyrene, prevalent in human ultra-processed foods (Adeyeye & Ashaolu, 2021; Dasa et al., 2022; Geng et al., 2024; Perera et al., 2021). Many of these compounds result from the Maillard Reaction (MR) and have been found in thermally processed pet foods, though research is scarce compared to human studies (Beynen; Bridglalsingh et al., 2024; Montegiove et al., 2020; Montegiove et al., 2023; Oba et al., 2022; Sugita et al., 2021; van Rooijen, 2015).

2.4 Maillard Reaction & Advanced Glycation End Products

Maillard Reaction

The Maillard Reaction (MR), characterized over a century ago, is a nonenzymatic browning reaction between reducing sugars and amino groups (Maillard). It produces flavor, coloring, and aromas resulting from thermal processing of food (exogenous MR) (Dyer et al., 1991; Starowicz & Zieliński, 2019). Endogenous MR occurs within an organism and can be conceptualized as a much lower and slower cooking cycle spanning over an organism's lifetime (Dyer et al., 1991). Exogenous and endogenous MR pathways generate AGEs and other compounds associated with inflammatory, degenerative and chronic diseases (Chaudhuri et al., 2018; Chen et al., 2018; Starowicz & Zieliński, 2019). Although associations with adverse health implications exist, MR is regarded as an essential reaction lending to food palatability and acceptance and increased antioxidative and antimicrobial properties (Hiramoto et al., 2004; Natella et al., 2002; Starowicz & Zieliński, 2019). These attributes arise from MR products giving foods aroma, flavor, and texture (Yu et al., 2020). Positive and negative attributes of the MR demonstrate the complexity of MRPs.

AGEs represent a heterogenous class of compounds resulting from non-enzymatic reactions between reducing sugars and proteins or nucleic acids. These modifications occur

endogenously during normal metabolism and exogenously during thermal processing of dietary ingredients (Poulsen et al., 2013). AGEs are of interest due to their presence in ultra-processed foods and their potential roles in numerous conditions (Chaudhuri et al., 2018).

The Maillard reaction produces AGEs, which proceed through early, intermediate and advanced stages, which generates intermediate compounds, Amadori products, and end products that may accumulate in tissues (Maillard, 1912). AGEs are diverse compounds and include fluorescent and non-fluorescent compounds making quantitation challenging. CML and CEL are among the most studied AGEs and have been proposed as biomarkers of disease progression (Nin et al., 2011). Research has mapped their formation pathways, tissue-specific distribution, and clearance mechanisms (Kawabata et al., 2011; Schleicher et al., 1997; Teerlink et al., 2004), topics covered in earlier sections.

Investigating AGE formation, metabolism and biological consequences has gained popularity as evidence supports their involvement in aging and disease; and their presence in human and commercial pet foods in addition to potential health implications highlights necessity for comparative research efforts. This is particularly important given commercial diets result in substantially higher levels of AGE intake on a metabolic body weight basis compared to human diets (van Rooijen, 2015), although biological consequences of these compounds remains poorly understood in veterinary medicine.

2.4.2 Early & Intermediate Maillard Reaction Stages

AGE Precursor & AGE Formation

The MR includes early, intermediate, and late-stage reactions (Figure 2.1). The initial step of the MR occurs when free or protein-bound amino groups interact with reducing sugars exogenously within foods during thermal exposure or endogenously within metabolic processes (Thomas et

al., 2015). This initial early stage involves a nonenzymatic condensation reaction between carbonyl groups of a reducing sugar (e.g., glucose or fructose) and free amino groups of amino acids, proteins, or nucleotides producing Schiff bases (N-substituted glycosylamines) (Chen et al., 2018; Emel'yanov, 2016; Parisi & Luo, 2018; Twarda-Clapa et al., 2022). Typically, this reaction involves amine groups from lysine or arginine although it can include other amino acids. Resulting Schiff bases are unstable and reversible intermediates that undergo subsequent Amadori rearrangement (glucose-derived) or Heyns rearrangement (fructose-derived) generating more stable products. Amadori rearrangement yields ketosamines (e.g. fructolysine) and Heyns rearrangement forms ketohexosamines. These stable intermediates serve as precursors to AGEs (Emel'yanov, 2016; Khalid et al., 2022; Twarda-Clapa et al., 2022).

Intermediary Amadori and Heyns rearrangement products may enter pathways generating a variety of compounds including fission products, reductones, 5-hydroxymethylfurfural (5HMF), and furfural, or they may undergo rearrangement and degradation to yield reactive dicarbonyl compounds. Hemoglobin A1c, a biomarker of blood glucose concentrations over time with high levels indicative of impaired glucose metabolism in humans and dogs, is a notable example of an endogenous Amadori product (Wasim et al., 2022).Dicarbonyl compounds, including methylglyoxal (MG) and glyoxal (GO) are AGE precursors that contribute to oxidative stress (Chen et al., 2022; Gill et al., 2019; Khalid et al., 2022; Sergi et al., 2021). MG and GO may be metabolized to heterocyclic amines, acrylamides, and other low molecular weight (LMW) compounds. These intermediates can also undergo rearrangement forming AGEs such as Nε-Carboxymethyl-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL), methylglyoxal hydro imidazoline-1 (MG-H1), pyrrolidine, argpyrimidine (AP), pentosidine, and others. While precursors such as MG and GO can be produced from several pathways, AGEs are only formed

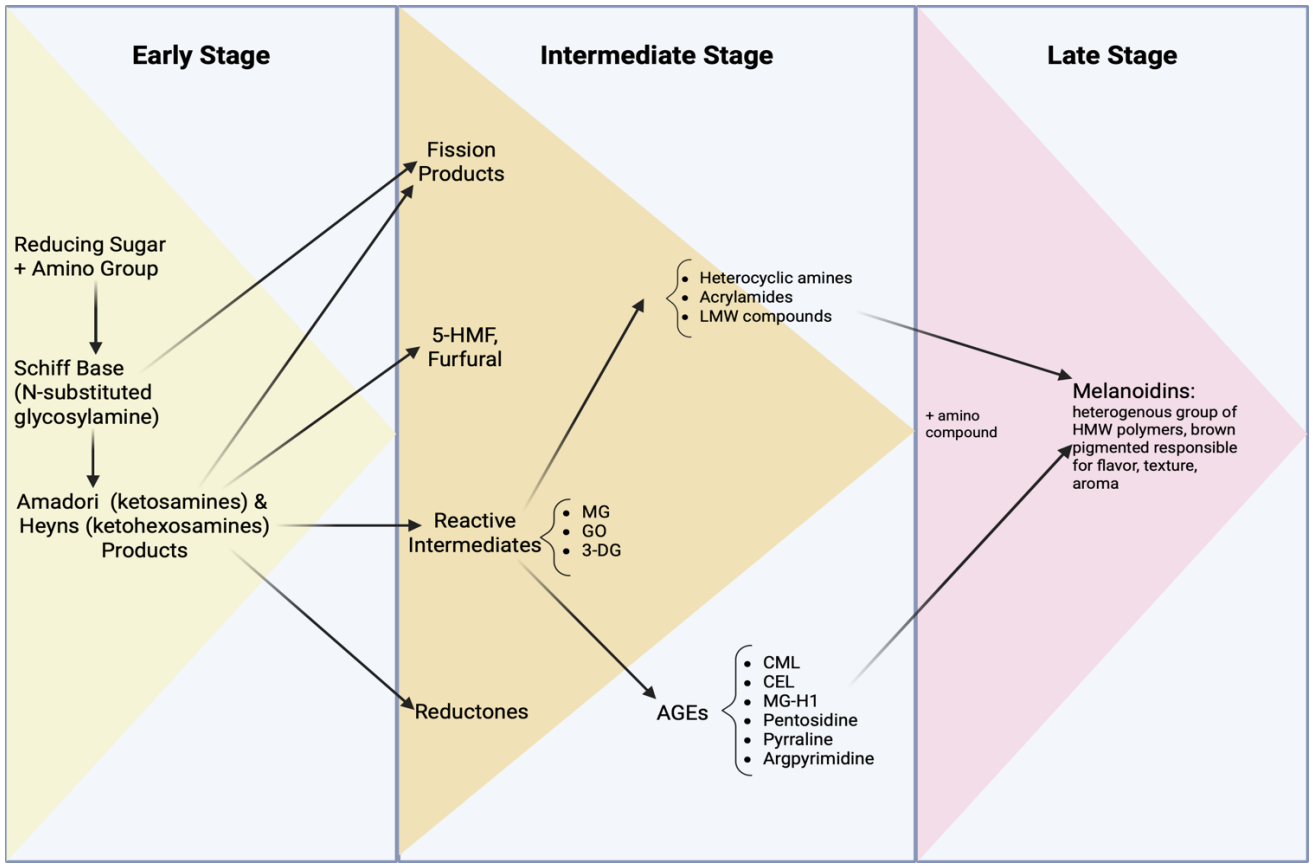

Figure 2.1. Stages of the Maillard Reaction (MR): Formation of Advanced Glycation End Products (AGEs) & Melanoidins. Early stages involve Schiff bases & Amadori/Heyns product formation. Intermediate stages generate reactive intermediates (e.g., methyl-glyoxal (MG), glyoxal (GO), 3-deoxyglucosone (3-DG)), reductones, fission products & AGE formation; heterocyclic amines, acrylamides & low molecular weight (LMW) compounds. Late stages produce high molecular weight (HMW) melanoidins, contributing to browning, flavor, & aroma. Created in https://BioRender.com

via the MR and associated Wolff, Namiki and Hodge Pathways (Figure 2.2). In this intermediate stage, AGEs and other MRPs accumulate from exogenous dAGEs and endogenous metabolic processes with production and accumulation exacerbated during metabolic stress (Twarda-Clapa et al., 2022). As a result, AGEs function as organic biomarkers reflecting glycation activity within foods and the body.

2.4.3 Advanced Stage of the MR

Melanoidin Formation

In the advanced stage of the MR, AGEs, heterocyclic amines, acrylamides, and other LMW compounds engage in additional reactions, some involving free amino groups (Figure 2.1). These interactions, influenced by antioxidant activity of some MRPs, form complex high molecular weight (HMW) polymers termed melanoidins (Langner & Rzeski, 2014) that are responsible for brown and caramelized colorings in thermally processed foods. The free amino groups involved in this reaction mainly originate from the side chains of lysine and arginine residues, which are susceptible to glycation and subsequent cross-linking reactions. Conditions promoting these reactions include high cooking temperatures, prolonged cooking times, multiple cooking steps, and the presence of reducing sugars, all of which are common in food processing and manufacturing.

Melanoidins have antioxidant properties and scavenge free radicals potentially inhibiting oxidative spoilage of foods (Brudzynski & Miotto, 2011), but also exacerbate inflammatory cascades and promote oxidative stress. These negative effects occur when reactive carbonyl groups of AGEs react with amino acid residues in body proteins, producing stable covalent bonds that alter protein structure and function including enzymatic catalysts, receptor signaling, hormone regulation, structural function and cellular signal transduction.

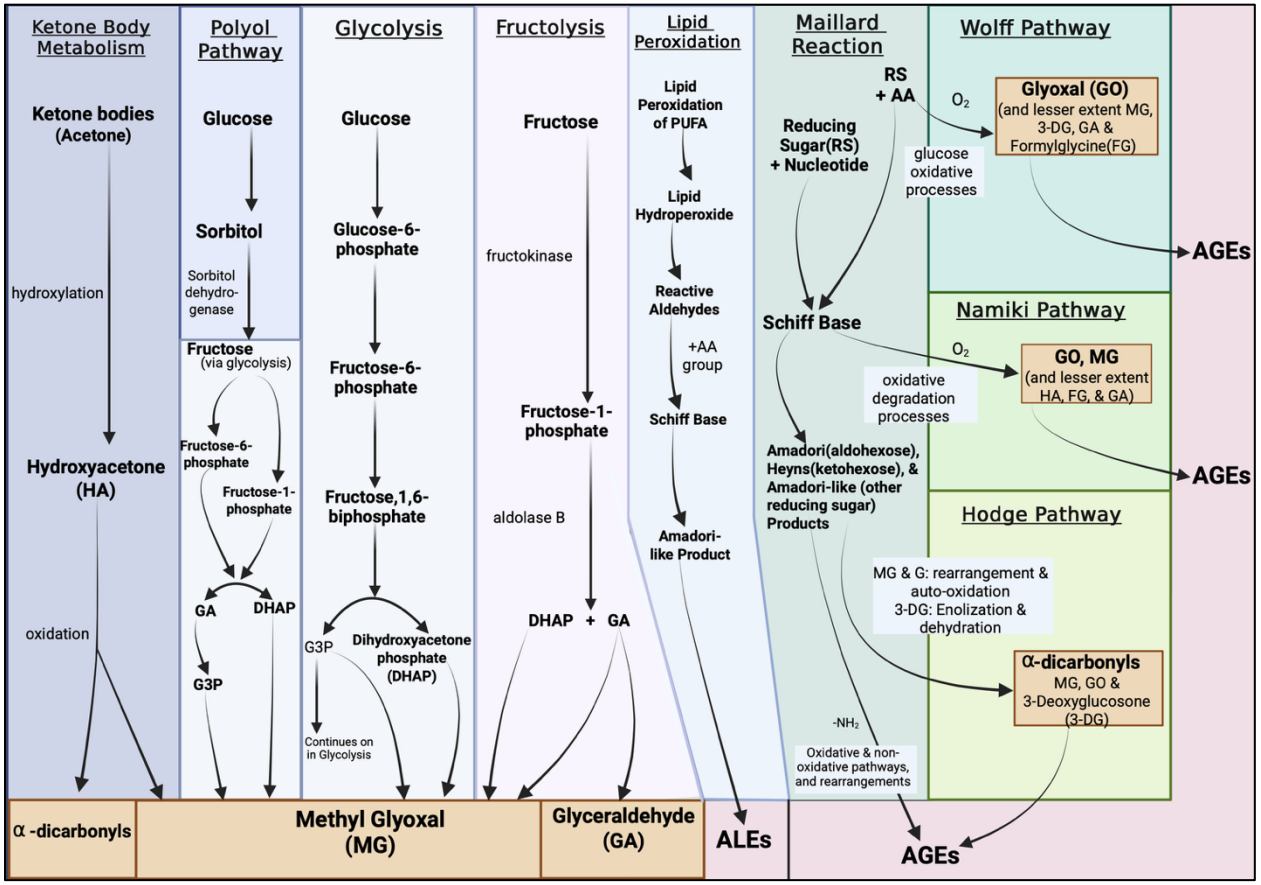

Figure 2.2 Pathways of Advanced Glycation End Product (AGE) and Precursor Formation: Metabolic and oxidative pathways contributing to AGE and α-dicarbonyl precursor formation. Pathways include glycolysis, fructolysis, lipid peroxidation, ketone body metabolism, the polyol pathway, and the Maillard reaction. The Wolff, Namiki, and Hodge pathways illustrate key oxidative and non-oxidative processes leading to AGE formation. Figure modified and expanded from Khalid, 2022 (Khalid et al., 2022) Created in https://BioRender.com

2.4.4 Alternative Pathways for Precursor & AGE Formation

In the initial stage of the MR, the reducing sugar, Schiff base, or Amadori product, may result in production of dicarbonyl compounds and AGEs (Figure 2.2) (Khalid et al., 2022; Twarda-Clapa et al., 2022). Reducing sugars may enter the Wolff Pathway (autoxidative glycosylation) pathway yielding the dicarbonyl, arabinose GO (Degenhardt et al., 2005). In the secondary stage, Schiff bases can enter the Namiki pathway and undergo reverse aldol oxidation and auto-oxidative cleavage producing dicarbonyls, such as GO and MG (Chen et al., 2022; Degenhardt et al., 2005; Khalid et al., 2022). In the later stages, Amadori products proceeding through the Hodge pathway are subject to rearrangement and auto-oxidation resulting in AGE precursors (Chen et al., 2022; Sergi et al., 2021). Alternatively, the initial reducing sugars, Schiff bases and Amadori products can proceed through conventional MR pathways, forming stable AGEs as described in figure 2.2.

AGE precursors, such as dicarbonyls, may also be produced from ketone body metabolism, glycolysis, and the polyol pathways (Figure 2.2) (Aragno & Mastrocola, 2017; Khalid et al., 2022). Stable AGE formation primarily occurs through two distinct mechanisms: the Hodge pathway and dicarbonyl reactions with lysine and arginine residues (Chen et al., 2022; Christiane Ott et al., 2014). Advanced Lipoxidation End Products (ALEs) are structurally similar to AGEs and share dicarbonyl precursor formation pathways but are members of a different group of compounds (Chen et al., 2022; Twarda-Clapa et al., 2022). Endogenous AGE and ALE formation increases during normal aging and during periods of oxidative stress, inflammation, and hyperglycemia, especially when detoxification capacity is overwhelmed (Dong et al., 2022; Dozio et al., 2023; Hooshiar et al., 2022; Khalid et al., 2022; Rungratanawanich et al., 2021; Sergi et al., 2021).

2.4.5 AGE Pool

The systemic AGE pool represents a sum of exogenous and endogenous AGEs and represents the overall burden of AGEs influencing health outcomes (Figure 2.3). Exogenous AGEs are predominately derived from processed foods subjected to high-thermal cooking methods such as grilling, roasting, or frying (Poulsen et al., 2013), tobacco smoke, ionizing radiation, air pollution and UV light (Nicholl et al., 1998; Perrone et al., 2020). Gastrointestinal absorption of dietary AGEs (dAGEs) contributes to the AGE pool (Koschinsky et al., 1997) and may induce deleterious systemic effects such as hyperglycemia, hyperlipidemia and exacerbated oxidative stress and inflammation (Del Turco & Basta, 2012; Liang et al., 2020). Endogenous AGE production rates are accelerated during periods of metabolic stress including hyperglycemia, oxidative stress, and more (Aragno & Mastrocola, 2017; Bierhaus et al., 2005; Chaudhuri et al., 2018; Dong et al., 2022; Dozio et al., 2023; Erusalimsky, 2021; Garay-Sevilla et al., 2020; Gasparotto et al.; Hooshiar et al., 2022).

Endogenous and exogenous AGEs induce structural and functional alterations in cartilage and connective tissues (Koschinsky et al., 1997; Liang et al., 2020), by reducing tissue elasticity through covalent cross-linking of collagen fibers and impairing normal physiological function contributing to age-related conditions like osteoarthritis (Delrue et al., 2023). Endogenous AGEs are associated with progressive non-enzymatic alteration of ‘browning’ of aging tissues (Verzijl et al., 2000). AGE-mediated glycation and cross-linking occurs with other structural and functional proteins including elastin (Konova et al., 2004), myelin (Vlassara et al., 1984), fibronectin (Tarsio et al., 1985), tubulin (Williams et al., 1982), crystallin (Luthra & Balasubramanian, 1993), laminin (Federoff et al., 1993), actin (Pekiner et al., 1993), myofibrillar

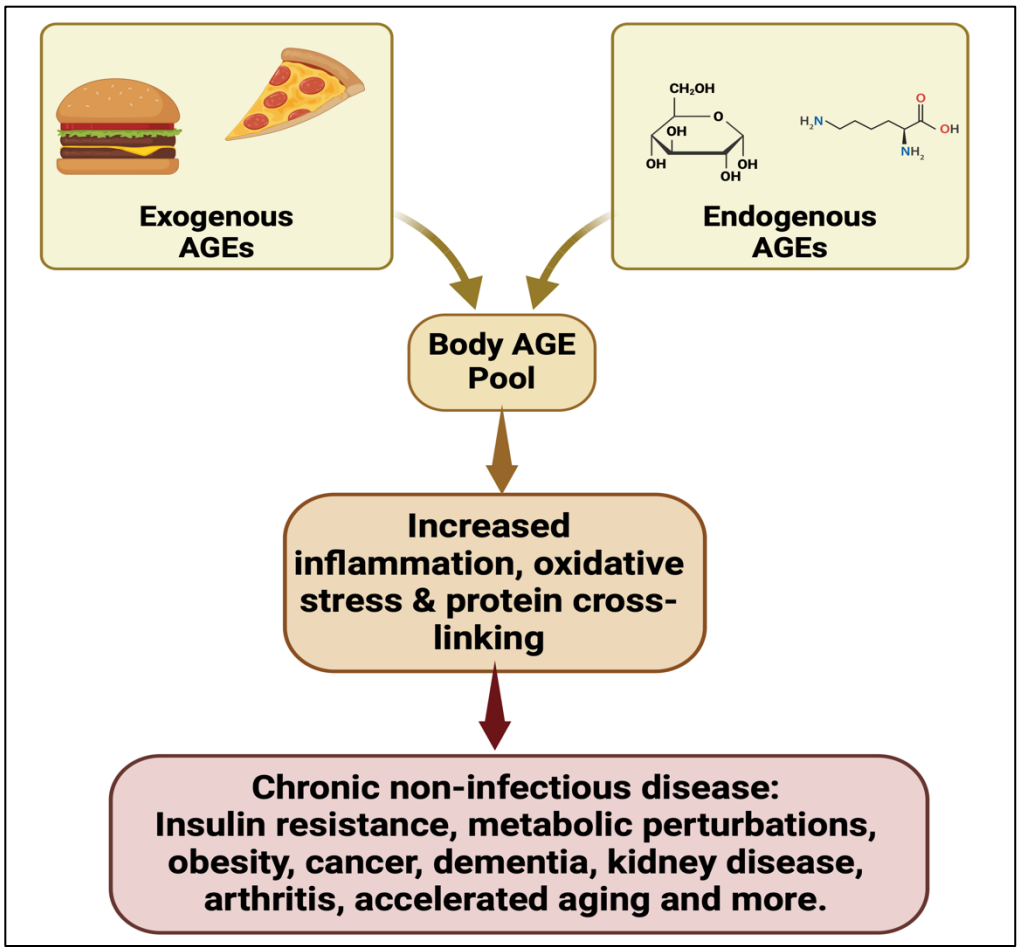

Figure 2.3 Body AGE pool, a combination of exogenous and endogenous AGEs. Created in https://BioRender.com

proteins (Syrovy & Hodny, 1993), hemoglobin (Dyer et al., 1993), lens membranes (Liang, 1993), albumin (Schleicher et al., 1993), LDL lipids (Bucala & Vlassara, 1995) and apoprotein (Bucala et al., 1993) indicating a role in related pathologies. Glycation and subsequent AGE formation and cross-linking disrupt protein-protein interactions, enzymatic function, and receptor binding, affecting cellular homeostasis and influencing pathogenesis of age-related and metabolic disorders (Chaudhuri et al., 2018). AGEs negatively affect low turnover, long-lived

matrix proteins; however, they also effect higher turnover compounds contradicting the common misconception that AGEs have minimal negative effect on high-turnover tissues (Ansari & Dash, 2013; Dyer et al., 1993; Sensi et al., 1995; Yamagishi, 2011). Thus, AGEs also effect shorter-lived proteins resulting in negative pathophysiological consequences (Rondeau & Bourdon, 2011; Rungratanawanich et al., 2021).

2.4.6 AGE Categorization

Over 20 AGEs have been detected within human biofluids, tissues, and food products; their categorization is reviewed elsewhere in detail (Perrone et al., 2020; Twarda-Clapa et al., 2022). AGEs are commonly categorized into four main groups based on chemical structure and fluorescence (Table 2.2).

Alternatively, AGEs may be classified by source (e.g. endogenous versus exogenous), or as LMW (AGE bound to a peptide or unbound) and HMW (AGE bound to protein). AGEs also may be characterized by toxicity (TAGEs) since binding with the receptor for AGE (RAGE) induces inflammatory signaling reactions that are cytotoxic (Takeuchi et al., 2001). Although categorization by toxicity assumes that some AGEs induce post-translational modifications or exert protective roles during times of surplus (Twarda-Clapa et al., 2022), however the roles and biological fates of many AGEs are less understood (Hellwig et al., 2024). As such, categorizing AGEs based on toxicity alone has limitations.

Precursors involved in formation offers an additional method for classification: including glucose-derived (Glu-AGEs), fructose-derived (Fru-AGEs), glycolaldehyde-derived (Glycol-AGEs), glyceraldehyde-derived (Glycer-AGEs), methylglyoxal-derived (MGO-AGEs), glyoxal-derived (GO-AGEs), and 3-deoxyglucosone-derived (3DG-AGEs) (Twarda-Clapa et al., 2022), that is useful in food processing research. This approach aids in identifying formation pathways,

informs mitigation strategies, refines analytical methods, and aids in understanding roles in disease (Inan-Eroglu et al., 2020; Patel et al., 2024; Perrone et al., 2020).

Table 2.2 Classification of Advanced Glycation End Products (AGEs) by Fluorescence and Cross-Linking Properties. Examples highlight well-characterized AGEs commonly studied in biological systems and food matrices.

Category	Description	Examples
Fluorescent and cross linked	Typically form on extracellular matrix proteins (e.g., collagen, elastin, lens crystallin, albumin) through ribose-mediated glycation of lysine or arginine residues. Pentosidine one of most well characterized (Singh et al., 2001) and biomarker estimating total AGE accumulation	Pentosidine, pentodilysine, crossline, AGE'XI, vesperlysine A and C, fluorescent fatty acid-pyrrole compound **(**FFPC)
Fluorescent and non-cross linked	Form through glycation but remain monoadducts without inducing protein cross-linking.	Argpyrimidine (AP)
Nonfluorescent and cross linked	Highly reactive, resulting in protein cross-linking and subsequent structural damage, tissue stiffness	Crossline, glyoxal lysine dimer (GOLD), methylglyoxal lysine dimer (MOLD), Deoxyglucosone-lysine dimer (DOLD), imidazolium cross-link derived from methylglyoxal and lysine-lysine (MODIC), and imidazolium cross-link derived from glyoxal, lysine-arginine (GODIC), Nδ-(5-Hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), Nδ-(2-hydroxy-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H2), Nδ-(2-methyl-5-hydroxy-4-imidazolon-2-yl)-ornithine (MG-H3)
Nonfluorescent and non-cross linked	Typically, non-cross-linking monoadducts. Common utilized as biomarkers due to abundance and detectability; most well-characterized category.	CEL, CML, pyrraline, pyrraline imidazolones

2.5 Physiological AGE Effects

AGEs interact physiologically through two distinct mechanisms: receptor-mediated binding and protein cross-linking (Gautieri et al., 2014; Reigle et al., 2008). Traditionally, cross-linking of long-lived structural proteins such as collagen has been noted due to its role in tissue stiffening and impaired elasticity. However, more recent evidence demonstrates that AGEs also form adducts on short-lived proteins altering their function, turnover and capacity for cellular signaling as discussed in an earlier section. These modifications can disrupt enzyme activity, impair receptor function, and contribute to metabolic dysfunction. Receptor mediated effects, through receptors like RAGE, modulate inflammation, oxidative stress, and immune responses, further influencing tissue remodeling and systematic disease risk (Chaudhuri et al., 2018; Fotheringham et al., 2022).

While AGE and associated receptor interactions are frequently linked to pathological outcomes, their effects vary by AGE subtype, receptor isoform, tissue context, and disease state. Emerging evidence suggests certain receptors (e.g. AGER-1) may exert protective effects by promoting AGE clearance and downregulating inflammatory signaling (Vlassara et al., 2009). A recent review emphasized that despite widespread associations between dietary AGEs and adverse health outcomes, a definitive causal role in humans remains ambiguous due to methodological limitations and inter-individual variability (Hellwig et al., 2024). Furthermore, the authors state that much of the mechanistic evidence is derived from rodent models (Hellwig et al., 2024), which leaves knowledge gaps in humans and dogs regarding receptor expression, signaling outcomes, and physiological relevance.

2.5.2 Protein Cross-Linking & Aggregation

In humans with diabetes mellitus, chronic hyperglycemia increases the formation of AGEs due to sustained elevations in circulating reducing sugars, particularly glucose. An early quantifiable product of this is hemoglobin A1c (HbA1c), formed by non-enzymatic glycation of hemoglobin to produce an Amadori product. HbA1c reflects mean blood glucose concentrations over the preceding 2–3 months and widely used as a diagnostic and monitoring tool for diabetes mellitus and pre-diabetic humans. Additionally, HbA1c can generate AGEs, contributing to the cumulative AGE burden associated with metabolic disease.

AGEs derived from HbA1c and other pathways can induce structural modification via cross-linking of proteins affecting protein function, especially those with slow turnover. In diabetic conditions, this predominantly affects collagen and low-density lipoprotein (LDL), contributing to arterial stiffness and LDL receptor binding impairment (Poznyak et al., 2023). Resultant increase in circulating LDL, cumulative AGE accumulation, and protein cross-linking perpetuates inflammation, oxidative stress, and reactive oxygen species (ROS) production likely contributing to progressive vascular, renal, neural, and ocular damage.

AGEs also induce protein aggregation associated with amyloidosis (Chellappa et al., 2020). Elevated argpyrimidine has been observed in familial amyloidosis protein aggregates suggesting the MR's role in pathogenic protein aggregation (Gomes et al., 2005). Similar mechanisms may contribute to neurodegenerative disorders, including Alzheimer’s disease, where AGE mediated protein misfolding and aggregation contributes to pathogenesis (Gasparotto et al.; Reddy et al., 2023).

2.5.3 AGE & Receptor Binding

AGEs interact with various cellular surface receptors, the most well-characterized being RAGE. Ligand binding to RAGE initiates intracellular signaling cascades involving nuclear factor kappa-B (NF-κB) activation, inflammatory cytokine production, and ROS generation. However, RAGE signaling is context-dependent and influenced by isoform expression, receptor density, and ligand type. Alternative receptors such as AGER1 (OST-48) appear to exert protective effects by facilitating AGE clearance and modulating inflammatory signaling. This contrast suggests that AGE-receptor interactions may be either detrimental or regulatory, depending on receptor expression profile, ligand characteristics, and disease context.

Excess ROS from RAGE activation contribute to increased AGE formation perpetuating oxidation of Amadori products and free sugars producing highly reactive dicarbonyl compounds including GO, MG, and 3-DG. Reactive dicarbonyls subsequently form irreversible AGEs such as CML, CEL, and crosslinked AGEs. As reactive dicarbonyl levels increase, particularly MG and GO availability of antioxidant defenses such as superoxide dismutase, catalase, glutathione and ascorbic acid, decrease (Chen et al., 2018). Oxidative stress and AGE formation activate inflammatory signaling pathways, such as NF-kB, increasing transcription of proinflammatory genes, mitogen-activated protein kinases (MAPK), and other transducers and activators of inflammatory, thrombogenic, and apoptotic reactions (Daffu et al., 2013; Uribarri et al., 2007; Younessi & Yoonessi, 2011). These events perpetuate oxidative stress and ROS production leading to further damage.

Human research of AGE/RAGE interaction and diet demonstrates complex physiological effects; however, knowledge gaps persist in dogs. Associations exist between AGE/RAGE and pathological conditions, including diabetes mellitus, cardiovascular disease, chronic kidney

disease, and neurodegenerative disorders in humans (Ahmed, 2005; Dong et al., 2022; Fotheringham et al., 2022; Juranek et al., 2015). Characterization of AGE/RAGE interactions spans molecular to systemic levels, including receptor expression patterns, activation of signaling pathways, and resulting physiological outcomes such as inflammation and oxidative stress. RAGE genes, RAGE structure, and RAGE-mediated pathways are conserved between humans and dogs. Alternative splicing of flRAGE occurs between species (Sterenczak et al., 2013). Similar conservation occurs in other species of mammals including rats, mice, and cattle (Murua Escobar et al., 2006), although humans and dogs demonstrate the highest similarity, also suggesting similar RAGE-mediated disease mechanisms. Additional studies demonstrate GI RAGE and associated isoform dysregulation in dogs with chronic enteropathies (A. Cabrera-García et al., 2021) and other canine conditions, including canine atherosclerosis, aging, osteoarthrosis, IBS, and certain cancers, and others (A. Cabrera-García et al., 2021; Comazzi et al., 2008; DeGroot et al., 2004; Heilmann et al., 2014; STERENCZAK et al., 2010; Weber et al., 1998). While RAGE genes between humans and dogs are highly conserved, the distribution, expression patterns and physiological responses species-specific considerations for disease management and therapeutic strategies.

2.5.3.1 Receptor for AGEs (RAGE)

AGEs bind to various receptors, the most common and well-characterized one is multi-ligand pattern recognition receptor for AGEs (RAGE), (Neeper et al., 1992). RAGE is a member of the immunoglobulin superfamily of receptors, functioning as a multi-ligand, cell surface pattern-recognition receptor. It shares ligands and signaling pathways with the toll-like receptor family (TLR), which are involved in immune and inflammatory pathway regulation (Sparvero et al., 2009). The most common form of RAGE is the canonical form known as full length membrane

bound receptor (mRAGE or flRAGE). This is the primary functional receptor involved in ligand binding and intracellular signaling. RAGE has three distinct parts: extracellular, transmembrane, and intracellular segments. The ligand binding site, or extracellular site, consists of three immunoglobulin domains, two C-type domains, and one V-type domain (Sparvero et al., 2009). The transmembrane portion is followed by the highly charged intracellular segment, associated with signaling and inflammatory cascades seen in many disease states (Dong et al., 2022).

RAGE is found in many tissues including vascular endothelial cells, immune cells, macrophages, neurons, cardiomyocytes, adipocytes, glomerular epithelial cells, podocytes, alveolar epithelial cells, and others (Dong et al., 2022). Located on the cell surface, RAGE binds AGEs and other ligands, including high mobility group box 1 (HMGB1), S100 proteins, lysophosphatidic acids, and amyloid beta, among others (Bierhaus et al., 2005; Buckley & Ehrhardt, 2010; Dong et al., 2022). Genetic polymorphisms within the RAGE gene influences transcriptional activity and ligand binding affinity, with some genetic variants increasing susceptibility to chronic disease development (Jangde et al., 2020; Sterenczak et al., 2013). To date at least 33 RAGE polymorphisms have been described in humans (Chaurasiya et al., 2023). Additionally, alternative splicing of RAGE mRNA generates multiple isoforms with distinct biological functions. At least 19 variants, termed RAGEv1-19, have been described in humans (Chaurasiya et al., 2023; Hudson et al., 2008; Rojas et al., 2024; Sterenczak et al., 2013). In dogs at least 24 naturally occurring RAGE splicing variants have been characterized (Murua Escobar et al., 2006; Sterenczak et al., 2009). Full-length RAGE-ligand binding and subsequent cascade of reactions are linked to diseases such as obesity, inflammation, cancers, diabetes, vascular and cardiac disease, neurodegenerative disorders, and more (Reddy et al., 2023). RAGE expression and splicing patterns are dynamically altered in response to disease, with certain isoforms

upregulated during chronic inflammation, cancer, and vascular diseases (Kalea et al., 2011). The shift increases production of flRAGE, enhancing cellular sensitivity to AGEs and increasing inflammatory signaling (Kalea et al., 2011). Consequently, RAGE dysregulation in disease states may exacerbate tissue damage by amplifying AGE-RAGE interactions and downstream oxidative stress. Outcomes of RAGE-ligand binding are determined by genetic factors, tissue, or organ location, the intra- or extra-cellular position of RAGE, and specific ligand binding among others.

RAGE expression is upregulated in response to ligand accumulation and activation of transcription factors, such as NF-κB, that drive its transcription (Bierhaus et al., 2005). A cascade of reactions occurs when AGEs or other ligands bind with RAGE. Such reactions induce oxidative stress, inflammation, and downregulation of mechanisms that protect from further protein glycation and carbonyl stress (Wasim et al., 2022). Binding activates various signaling pathways, including nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), and Janus kinase/signal transducers and activators of transcription (JAK/STAT), perpetuating inflammatory response (Khalid et al., 2022). Consequently, chronic RAGE activation by AGEs contributes to progression of inflammatory and degenerative diseases, such as diabetes, cardiovascular disease, and neurodegenerative disorders (Khalid et al., 2022; Reddy et al., 2023; Wasim et al., 2022).

In embryonic development RAGE expression is generally high, decreasing with age in most tissues except skin and lung (Buckley & Ehrhardt, 2010; Piras et al., 2016). RAGE expression increases above basal levels in various tissues as consequence of many chronic diseases due to stimulation by disease-associated ligands (Buckley & Ehrhardt, 2010; Dong et al., 2022). Ligands interacting with pattern-recognition receptors that detect microbial products

are classified as pathogen-associated molecular patterns (PAMPs), while those binding to endogenous molecules, called alarmins, are upregulated during tissue damage and inflammation are classified as danger-associated molecular patterns (DAMPs) (Jang et al., 2020). This suggests RAGEs have a role in innate immunity, contributing to immune responses recognizing external pathogens and internal signals of cellular distress (Kierdorf & Fritz, 2013).

2.5.3.2 RAGE Isoforms

Additional characterized RAGE isoforms include: soluble RAGE (sRAGE), endogenously secreted RAGE (esRAGE), cleaved RAGE (cRAGE), and dominant-negative RAGE (dnRAGE) (Figure 2.4). RAGE isoforms bind to RAGE ligands, inhibiting flRAGE interaction. Each receptor's role may differ based on tissue expression and disease state. sRAGE, esRAGE, and cRAGE isoforms are considered decoy receptors potentially reducing inflammation, while other isoforms contribute to inflammation (Buckley & Ehrhardt, 2010; Chellappa et al., 2020; Khalid & Adem, 2024).

sRAGE includes the collective pool of esRAGE and cRAGE unanchored to cell membranes and found within tissue extracellular environments (Prasad et al., 2016). cRAGE and esRAGE are C-truncated isoforms of fl-RAGE produced from different mechanisms. cRAGE results from proteolytic cleavage at the cell membrane, and esRAGE is secreted from alternative mRNA splicing at axon 9 (Figure 2.3) (Braley et al., 2016; Gasparotto et al.; Hanford et al., 2004; Kanikowska et al., 2024; Khalid & Adem, 2024; Prasad et al., 2016; Yonekura et al., 2003). sRAGE isoforms are considered decoy receptors for RAGE ligands potentially reducing inflammatory effects (Rojas et al., 2024; Yang et al., 2018). C-truncated forms of esRAGE and cRAGE, the most widely researched, are altered in various RAGE-associated human diseases (Khalid & Adem, 2024; Sterenczak et al., 2013). RAGE isoforms are often oversimplified

especially sRAGE where esRAGE and cRAGE are not distinguished. Measurement of sRAGE, commonly achieved with ELISA kits, specifically sRAGE and esRAGE-specific kits. Since total sRAGE includes cRAGE and esRAGE, subtraction of esRAGE from total sRAGE yields cRAGE (Prasad et al., 2016). Alternative RAGE detection methods, including western blot, fluorescence, and mass spectrometry, are available each with strengths and limitations (Corica et al., 2022).

Another isoform, dominant negative RAGE (dnRAGE), lacks the intracellular domain of fl-RAGE. This truncated isoform preserves transmembrane and extracellular portions of RAGE (Khalid & Adem, 2024). Over 19 naturally occurring RAGE splicing variants have been characterized at mRNA and protein levels, each lending potential variability of binding affinity for RAGE isoforms dependent upon truncation location (Khalid & Adem, 2024; Sterenczak et al., 2013).

Varying expression levels of circulating sRAGE have been identified in numerous diseases and explored as a potential biomarker for disease diagnosis, management, and/or severity; (A. I. Cabrera-García et al., 2021; Kanikowska et al., 2024; Lee & Park, 2013; Murua Escobar et al., 2006) however, expression levels differ among disease states. For example, low levels of sRAGE and esRAGE have been observed in coronary artery disease (CAD), hypercholesterolemia, chronic obstructive pulmonary disease, Alzheimer's disease, obesity, and others (Chiappalupi et al., 2021; Geroldi et al., 2005; Prasad, 2019). Alternatively, higher levels have been found in diabetes mellitus, chronic kidney disease, and some CAD patients (Dozio et al., 2023; Dozio et al., 2020; Hudson et al., 2005; Nin et al., 2011). Based on sRAGE's role as a decoy receptor, it is hypothesized that high expressed sRAGE levels would protect from RAGE expression; however, this would likely not be true in instances where AGEs and other RAGE ligands exceed sRAGE

capacity (Prasad et al., 2016). Thus, further research on RAGE expression, its isoforms, and circulating levels of free and protein-bound AGEs is required.

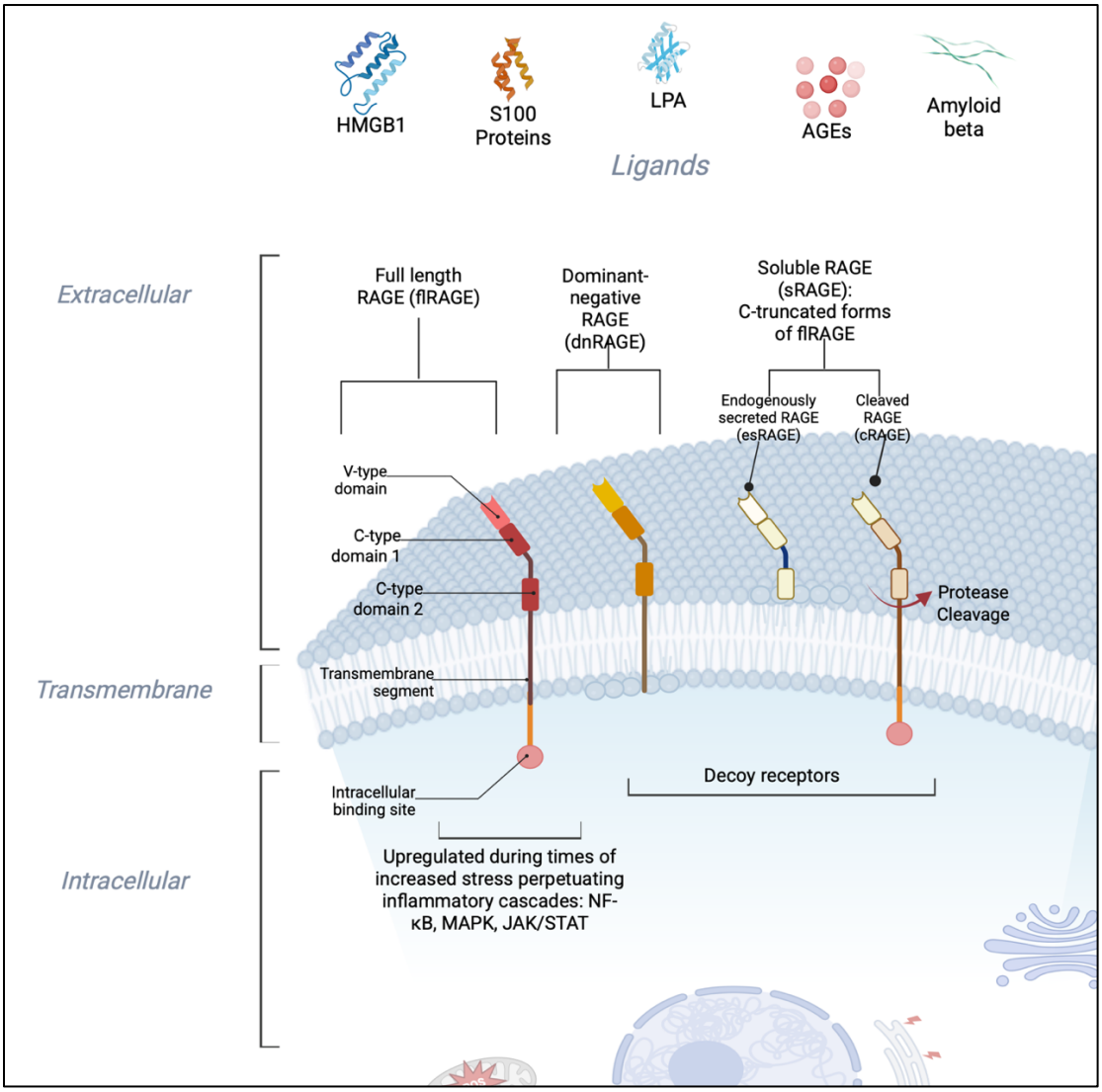

Figure 2.4 RAGE isoforms and structural characteristics: Full-length RAGE (flRAGE) contains all domains for signal transduction. Soluble RAGE (sRAGE), comprising cleaved RAGE (cRAGE) & endogenously secreted RAGE (esRAGE), & dominant-negative RAGE (dnRAGE) act as a decoy receptors. Figure adapted from (Khalid & Adem, 2024), Created in https://BioRender.com

2.5.3.3 Nuclear RAGE

Nuclear RAGE (nRAGE) is found in the cell nucleus and plays an important role in DNA repair, though mechanisms are poorly understood (Kumar et al., 2020; Kumar et al., 2017). Thus, intra- or extra- cellular location of RAGE influences whether there are inflammatory or protective roles. This nRAGE isoform localizes to the nucleus and modulates DNA repair via ATM-dependent phosphorylation. This mechanism has been shown to prevent damage associated with cellular senescence, inflammation, pulmonary fibrosis, and cancer (Kumar et al., 2017).

2.5.3.4 AGER Complex

AGEs interact with additional receptors, triggering diverse biological responses, some that may mediate adverse effects of AGEs (Prasad & Mishra, 2018). Downregulation of the AGER complex, comprised of AGER1, AGER2, and AGER3, is associated with disease development (Li et al., 1996; Vlassara et al., 1995). AGE receptor-1 (AGER1, or OST-48) is thought to function primarily as a scavenger receptor promoting detoxification and clearance of AGEs from the body (Vlassara et al., 2009). AGER1 activation enhances cellular defense mechanisms, upregulating antioxidant enzymes, and reducing formation of ROS and oxidative stress. AGER1 can be downregulated by high dAGEs and diabetes mellitus (Cai et al., 2006; He et al., 2000). AGE receptor-2 (AGER2) is less understood, but potentially involved in phosphorylation of protein kinase C on tyrosine residues, playing a role in intracellular AGE transport (Christiane Ott et al., 2014). AGER2 influences the GLUT4 trafficking pathway enhancing glucose transport (Hodgkinson et al., 2005). Additionally, AGER2 increases AGE signaling inducing cell activation and cytokine growth factor leading to adverse effects including metabolic perturbation (Pasupulati & Chitra; Stitt et al., 1997). AGE Receptor-3 (AGER3 or galectin-3), a member of the lectin family, has high RAGE ligand affinity (C. Ott et al., 2014; Vlassara et al., 1995). It is

involved with AGE removal and mitigating oxidative stress and inflammation. AGER3 is found mainly in the cell cytoplasm, although found in lesser concentrations in nuclei, on cell surfaces, and in extracellular spaces. AGER3 influences cell migration, adhesion, immune response modulation, and cancer metastasis (C. Ott et al., 2014).

2.5.3.5 Toll-Like Receptors

TLRs, (e.g., TLR2 and TLR4) are expressed on dendritic cells, macrophages, and non-immune cells, activating inflammatory pathways (van Beijnum et al., 2008). TLRs recognize AGEs as PAMPS or DAMPs, and upon binding activate intracellular pathways resulting in NF-kB activation and production of pro-inflammatory cytokines. Consequences of TLRs interacting with RAGE and associated ligands likely varies between cell types (van Beijnum et al., 2008).

2.5.3.6 Macrophage and Scavenger Receptors & More

Additional receptors involved in AGE uptake and clearance include macrophage scavenger receptor class A (MSR-1), various class B scavenger receptors (e.g., CD36 and SR-B1) and others (Miyazaki et al., 2002). AGE binding to these receptors results in degradation of AGE-modified proteins, preserving cellular and tissue function preventing AGE accumulation. SR-B1 also binds AGEs and oxidized lipoproteins clearing circulating modified proteins and affecting lipid metabolism (Miyazaki et al., 2002). The interplay between macrophages and AGEs demonstrates the role of the innate immune system in minimizing AGE-associated damage and chronic disease.

Although Table 2.3 presents a detailed list of AGE receptors, it is not exhaustive. These receptors and their roles demonstrate complexity of potential AGE-receptor interactions and metabolic outcomes. They also demonstrate the need for additional research on detailed

mechanisms of their respective pathways as well as other known and unknown AGE-receptor interactions.

Receptor	AGEs Binding	Location	Health Influence	Effects
RAGE	Multiple AGEs (e.g., CML, CEL, pentosidine)	Endothelial cells, smooth muscle cells, neurons, immune cells	Negative	Activates NF-κB, MAPKs, JAK/STAT pathways; promotes inflammation, oxidative stress, contributes to diabetic complications, atherosclerosis, neurodegenerative disorders.
sRAGE	Multiple AGEs	Circulation	Positive	Decoy receptor, preventing AGE/RAGE interaction; reduces inflammation, oxidative stress.
esRAGE	Multiple AGEs	Circulation	Positive	Decoy receptor, protects against AGE-related damage.
AGER-1 (OST-48)	Multiple AGEs	Various tissues	Positive	Involved in detoxification and clearance of AGEs; reduces oxidative stress, inflammation.
AGER-2 (80K-H)	Less defined, various AGEs	Various tissues	Both	Modulates cellular responses to AGEs; specific functions and pathways are not well-defined.
AGER-3 (Galectin-3)	β-galactoside sugars on glycated proteins	Immune cells, fibroblasts, epithelial cells	Both	Modulates immune responses and fibrosis; linked to cardiac fibrosis, heart failure, and chronic kidney disease.
Toll-like Receptors (TLR2, TLR4)	Multiple AGEs	Immune cells (macrophages, dendritic cells), non-immune cells	Negative	Activation of inflammatory responses via MyD88-dependent pathway; contributes to atherosclerosis, diabetic complications.
Macrophage Scavenger Receptors (MSR-1, CD36, SR-B1)	AGE-modified proteins and lipoproteins	Macrophages, other phagocytic cells	Both	Facilitate endocytosis and clearance of AGEs; involved in foam cell formation and plaque development in atherosclerosis.

Table 2.3 AGE Receptors (KEY: Receptor for AGEs (RAGE), Nuclear Factor kappa-B (NF-κB), Mitogen-Activated Protein Kinases (MAPKs), Janus Kinase/Signal Transducers & Activators of Transcription (JAK/STAT), AGE Receptor 1 (AGER-1), Oligosaccharyltransferase-48 (OST-48), AGE Receptor 2 (AGE-R2), 80 Kilodalton Heat shock protein (80K-H), AGE Receptor 3 (AGE-R3, or Galectin-3), Toll-Like Receptor (TLR), Macrophage Scavenger Receptor 1 (MSR-1), Scavenger Receptor Class B Type 1 (SR-B1), Cluster of Differentiation 36 (CD36), Soluble Receptor for AGEs (sRAGE), Endogenous Secretory Receptor for AGEs (esRAGE))

2.6 Metabolic Fates of AGEs

Not all dAGEs are absorbed and become part of the AGE pool. It is estimated that ten to thirty percent of dAGEs are absorbed with approximately two-thirds of the absorbed AGEs remaining in the body (Koschinsky et al., 1997). As a result, dAGEs influence health and disease (Chen et al., 2022). The body's ability to detoxify and eliminate AGEs is an important factor in managing its AGE pool and mitigating oxidative stress. Several routes of AGE metabolism, absorption, detoxification, and elimination exist, including GI absorption, fecal elimination, urinary elimination, gut microbe metabolism and fermentation, and others; however, there is limited detailed evidence for each of these mechanisms.

2.6.1 Gastrointestinal Tract Absorption & Fecal Excretion

Heating protein results in denaturation, such as with thermal treatment of food. Likewise, AGEs produced with thermal treatment of food may become protein bound resulting in HMW protein-bound AGEs being trapped in the GI tract. HMW protein bound AGE's require gastric and intestinal peptidases to release the AGE bound to its respective free amino acid, di or tripeptide (Hellwig et al., 2014; Poulsen et al., 2013; Snelson & Coughlan, 2019). While the GI tract plays a role in absorption, metabolism, and excretion of dAGEs, mechanisms regarding bioavailability and metabolic fates of AGEs are controversial (Fotheringham et al., 2022). Ten to thirty percent of dAGEs are absorbed from the GI tract into circulation. (He et al., 1999; Koschinsky et al., 1997).

AGEs are absorbed via four routes: peptide transporter 1 (PEPT1), transcytosis, paracellular diffusion, and simple diffusion (Zhao et al., 2019). Simple diffusion is not effective as most AGEs remain within gut lumen (Twarda-Clapa et al., 2022). Dipeptide versions of CML, CEL, pyrraline, and MG-H1 are absorbed through intestinal epithelium via PEPT1 (Geissler et

al., 2010; Hellwig et al., 2011). Transcytosis facilitates uptake of HMW AGEs via vesicular transport across enterocytes. Paracellular diffusion passively moves LMW AGEs through tight junctions, especially in conditions with altered intestinal permeability. However, transcytosis and paracellular diffusion are largely inefficient, resulting in fecal excretion of unabsorbed AGEs. Some dAGEs, especially HMW AGEs, are eliminated in feces or fermented and metabolized by gastrointestinal (GI) microbes. Up to a third of dAGEs are eliminated in feces as estimated by CML intake and excretion (Roncero-Ramos et al., 2013). Protein-bound AGEs have limited absorption, but may impact gut barrier function by altering tight junction integrity, independent of inflammatory signaling (Jansen et al., 2023).

Different concentrations of dAGEs concentrations have been found throughout the GI tract. Specifically, varying levels of CML have been found between different GI tract segments (Qu et al., 2018). Intake of CML is associated with disordered microbiota due to perturbed metabolic pathways of carbohydrate, amino acid, and energy metabolism, as well as impaired protein fermentation in mice (Qu et al., 2018). However, mechanisms of general AGE absorption and fates of individual AGEs concerning degradation and elimination pathways within the GI tract and associated pathways in humans and dogs are not well understood.

2.6.2 Microbial Metabolism

Gut microbes have demonstrated ability to metabolize some dAGEs. Degradation of frutosyllysine, partial degradation of CML and up to 20% degradation of pyrraline have been observed over 24 hours (Hellwig et al., 2015). Certain microbial species anaerobically degrade CML into carboxymethylated biogenic amines and other carboxylic acids (Bui et al., 2019). Diet influences the microbiota and therefore metabolism of AGEs. Formula fed infants, for example, demonstrate microbiota ability to degrade this AGE, while breast fed infants do not. Presumably

the difference in microbial adaptation is attributed to dAGE exposure differences (Bui et al., 2020).

Microbial fates of each AGE is unlikely to be uniform, as specific AGEs have been shown to influence the amounts of certain microbial species (Snelson & Coughlan, 2019). While *in vivo* evidence is limited, one study showed dAGEs altered the human microbiome in a manner that is both dose-and microbe-dependent (Aschner et al., 2023). Consumption of a high dAGEs in adolescent males reduced *Lactobacilli* and increased *Enterobacteria*, shifting the microbial balance toward a more pro-inflammatory state without significantly altering *Bifidobacteria*, *Bacteroides*, *E. Rectale*, or *Clostridium leptum* (Seiquer et al., 2014). Similarly, in peritoneal dialysis patients, AGE restriction over one month led to a reduction in *Prevotella copri* and *Bifidobacterium animalis* and increased *Alistipes indistinctus*, *Clostridium hathewayi*, *Clostridium citroniae*, and *Ruminococcus gauvreauii* (Yacoub et al., 2017). These microbial changes could contribute to systemic effects via altered short-chain fatty acid production (SCFA), gut permeability, or low-grade inflammation (Aschner et al., 2023; Nogueira Silva Lima et al., 2024).

Diets that are high in dAGEs contain other compounds like melanoidins, which have prebiotic effects that can benefit various microbe species (Snelson & Coughlan, 2019). *In vitro* studies demonstrate increased production of *Bifidobacteria* from thermally treated bread crust, glycated gluten, and pea proteins, while *in vivo* studies in rats show reductions with similar diets (Borrelli & Fogliano, 2005; Delgado-Andrade et al., 2017). Diets supplemented with high-melanoidin bread crust, low-melanoidin bread crumb, or fiber-free bread resulted in similar increases of *Bifidobacteria* (Helou et al., 2017); thus, melanoidin content alone is not responsible for *Bifidobacteria* increases. Decreased inflammation and weight loss was found in mice fed

thermally treated diets in an induced colitis model; specifically, CML protected against weight loss and modulated a healthier microbiome (ALJahdali et al., 2017).

GI bacteria can ferment AGEs, producing SCFAs and other metabolites (Snelson & Coughlan, 2019). Several bacterial species produce butyrate, a SCFA and energy source for enterocytes (Fusco et al., 2023). Reduction of butyrate in the gut is associated with decreased epithelial barrier function and increased inflammation and oxidative stress (Wang et al., 2022). The gut microbiota play a role in maintaining intestinal barrier integrity (Dmytriv et al., 2024) preventing absorption of dAGEs and other compounds into circulation. An unhealthy or imbalanced gut microbiome, termed dysbiosis, compromises part of the intestinal barrier; increasing absorption of dAGEs, various compounds, and undigested food particles into circulation, contributing to systemic inflammation and metabolic disorders (Aleman et al., 2023). Dysbiosis and associated disease has come under scrutiny due to lack of clear definition, and oversimplification of the complex relationship between microbiota composition and host health (Brüssow, 2020). Thus, the field is substantially far from distinguishing cause from consequence in disease states due to a reliance on underpowered studies and models for human disease (Brüssow, 2020).

Interaction between AGEs and GI microbiota and modulating effects on the immune response of host are conflicting. Rodent models are used to investigate microbial generation of SCFA and AGEs; however, similar studies in other species including humans are lacking (Nagpal et al., 2018). Few *in vitro* studies have investigated dAGEs effect on SCFA production. Fecal samples from healthy humans and those with colitis fed different glycated proteins showed no change in SCFA levels (Dominika et al., 2011; Mills et al., 2008). Different glycated proteins have different effects on SCFA. SCFAs were reduced when subjects were fed glycated gluten

(Dell'Aquila et al., 2003). Collectively, rodent and *in vitro* studies emphasize the need for broader research to determine effect of dAGEs on GI health and microbial diversity in various diseases. Although relationships between different diet types and fecal SCFAs have been investigated, no *in vivo* human studies have investigated relationships between high dAGEs and fecal SCFA concentration. Although, some research suggests that adult fecal microbiota capability to degrade some AGES, such as frutosyllysine, to produce butyrate does demonstrate some age and diet dependent patterns (Bui et al., 2020).

2.6.3 Renal Metabolism

An estimated one-third of GI-absorbed dAGEs are excreted in urine within 48 hours in healthy humans (Koschinsky et al., 1997). In disease states, such as advanced CKD, kidneys have decreased capacity for AGE clearance from plasma leading to increased plasma AGE concentration and further damage (Makita et al., 1994). In humans with diabetic nephropathy or CKD, renal AGE excretion may be lower than 5% (Scheijen et al., 2018). Renal AGE metabolism is complex involving filtration and secretion. Endogenous and exogenous compounds such as glucose, amino acids, proteins, and metabolic products are filtered by the glomerulus. Many filtered substances are reabsorbed by the renal tubules while protein-bound molecules and certain toxins are actively secreted by renal tubules at varying rates. Circulating LMW AGEs and small AGE-modified proteins undergo glomerular filtration and may be reabsorbed by the renal tubules and absorbed or eliminated in the urine. Tubular cells absorb and degrade AGEs via the endolysosomal apparatus of the proximal convoluted tubule demonstrating the kidneys' role in AGE metabolism (Gugliucci & Bendayan, 1996; Saito et al., 2005). AGEs role in compromised kidney function has been reviewed, with studies demonstrating the complexity that AGEs have in disease (Busch et al., 2010; Dozio et al., 2023).

2.6.4 Hepatic Metabolism

The liver is the main catabolic site for circulating AGEs, specifically CML (Horiuchi, 2002; Šebeková et al., 2002), as opposed to the kidney (Horiuchi, 2002; Smedsrod et al., 1997). Plasma CML levels are elevated in cirrhotic patients suggesting correlation with disease severity when compared to healthy controls (Šebeková et al., 2002). CML levels decrease in patients following liver transplantation (Šebeková et al., 2002). Thus, plasma AGE levels may be biomarkers of liver function. Additional animal models show AGE-modified bovine serum albumin administered intravenously is taken up and cleared via liver endothelial, Kupffer, and parenchymal cells (Nagai et al., 2007; Smedsrod et al., 1997).

Collectively AGEs undergo first-pass metabolism primarily involving hepatocytes. It is less likely that hepatic phase I or II detoxification in the liver is involved in AGE clearance (Poulsen et al., 2013) since AGEs are water-soluble and enzymes involved in phase I metabolism are found within endoplasmic reticulum lipid membranes. Furthermore, phase II metabolism involves coupling reactions requiring side groups that are uncharacteristic of most AGEs. Competing theories for primary AGE metabolism sites could be attributed to differing affinity of AGEs in various tissues.

2.6.5 Tissue Clearance

Tissue specific AGE clearance involves multiple cellular and molecular mechanisms working collectively to manage AGE accumulation. Various tissues demonstrate distinct patterns of AGE accumulation and clearance. Skeletal muscle exhibits high AGE accumulation due to high protein content and relatively slow turnover rate (Olson et al., 2021). Cartilage and connective tissues show considerable AGE accumulation, particularly in aging and disease, attributable to

limited regenerative capacity and slow protein turnover (Gautieri, Passini, Silván, Guizar-Sicairos, Carimati, Volpi, Moretti, Schoenhuber, Redaelli, & Berli, 2017).

Cellular proteolytic mechanisms include receptor and non-receptor-mediated pathways metabolizing AGEs into peptides and releasing them back into circulation (Vlassara, 2001). Main clearance mechanisms include lysosomal degradation of AGE-modified proteins and proteasomal degradation of smaller AGE-modified proteins (Höhn et al., 2013). Autophagy-mediated clearance and receptor-mediated endocytosis of cellular components damaged by AGEs help limit AGE accumulation (Rajawat et al., 2009; Takahashi et al., 2017). Oxidative stress localized in tissues may influence AGE clearance rates, and increased AGEs and receptor expression can impact clearance capacity (Maldonado et al., 2023; Twarda-Clapa et al., 2022). Distribution and density of AGE receptors influence AGE clearance capacity and tissue-specific AGE accumulation response (Twarda-Clapa et al., 2022). Effectiveness of each mechanism depends upon multiple physiological factors including tissue type, metabolic and disease state, and biological age (Andersson et al., 2013; Liu et al., 2015; Takahashi et al., 2017).

2.7 AGE & RAGE Detection & Quantification

Although AGE formation chemistry has been known since the early twentieth century, modern AGE research began approximately 4 decades ago, with over 20 unique AGEs identified in human blood, tissues and food. Despite decades of research, reliable detection and quantification has not been achieved. Isolation of all AGEs has also not been realized and is still a barrier within the field. Obstacles include lack of universally accepted detection methods, widely accessible internal standards and standard units of measurement have hindered advancement (Singh et al., 2001), although standards have become available more recently for many AGEs

and associated compounds. Methodologies for AGE detection also lacks standardization, compromising comparison findings between laboratories and studies (Smit & Lutgers, 2004).

AGEs have broad ranges of physical properties, molecular polarity, molecular weights, and fluorescent properties, as discussed earlier. Because of this heterogeneity, no method can detect all known AGEs hindering investigations of AGEs and their role in health and disease (Perrone et al., 2020). Common methods for detecting AGEs include colorimetric assays, ELISA, MS, and NMR. Each methodology possesses strengths and limitations based on objective and AGEs targeted.

2.7.1 AGE Detection Methods

Fluorometric and autofluorescence spectroscopy methods are most commonly employed for AGE detection (Jaisson & Gillery, 2021). The fluorometric method detects fluorescent AGEs, specifically pentosidine, in serum, urine, and saliva, but is insensitive to nonfluorescent AGEs. Further, non-AGE fluorophores can interfere with compromising its reliability.

Autofluorescence spectroscopy detects and estimates total fluorescent AGEs *in vivo* skin tissue by measuring characteristics of fluorescence wavelengths. This technology, originally developed for Caucasians in The Netherlands, has limited applicability in other ethnicities due to interference from skin color with some wavelengths (Yamanaka et al., 2016).

ELISA has been widely used due to high throughput capacity, commercial availability, and affordability. The method is valued for simplicity, speed, and commercial availability, with kits available to target various types of AGEs. It typically measures protein-bound AGEs and excludes free AGEs. ELISAs focus on protein bound forms may overlook fluctuations in free AGEs reflecting dietary intake, renal clearance, or short-term metabolic response.

ELISA is often used to quantify serum, urine, and tissue AGEs based on antigen-antibody interactions. However, validity of results can be compromised by cross-reactions with other proteins (Corica et al., 2022; Perrone et al., 2020; Poulsen et al., 2013) and therefore considered semi-quantitative and should be analyzed with caution (Poulsen et al., 2013). Considering these points, ELISA testing for individual AGEs requires proper validation and characterization of antibodies and requires a separate kit for each AGE adding to logistical and cost considerations. Western blotting has also been utilized, but to a lesser extent. It provides tissue-specific information but is limited by the requirement of tissue samples. Results are varied based on antibodies used, and the variable nature of quantitative staining procedures (Corica et al., 2022; Perrone et al., 2020). More recent works have encouraged the use of specific analytical techniques for quantitative determination of AGEs, specifically GC-MS, LC-MS, and HPLC based methods (Ahmed et al., 2005; Charissou et al., 2007; Wellner et al., 2011).

Instrumental methods of AGE detection include gas chromatography (GC), liquid chromatography (LC), high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC). Each method can be coupled with mass spectrometry (MS) and tandem MS (MS/MS). While HPLC-based detection methods were historically predominant in literature, recent trends show UHPLC-based technologies becoming dominant (Perrone et al., 2020). GC-MS produces accurate results for AGEs such as CML and CEL in urine, although it is expensive and requires specialized training (Perrone et al., 2020). LC-MS/MS, is used for CML, CEL and MG determination in plasma and urine and requires specific training. HPLC requires extensive training due to complex processes, but can detect CML, CEL, MG, pentosidine, and other AGEs in biosamples. AGEs without a defined biochemical structure are not appropriate for HPLC detection methods. UHPLC is appropriate

for similar AGEs as HPLC and does not require knowledge of the complete structure. While more costly than HPLC and other methods and requiring trained personnel, it produces results with high resolution while being less resource sensitive (Perrone et al., 2020).

HPLC and LC-MS can separate and quantify multiple AGEs. They can be coupled with additional technologies such as GC or fluorescence detection to qualitate and quantitate AGEs. HPLC is commonly coupled with fluorescence for compounds like pentosidine, while LC-MS is more commonly used for broader ranges of AGEs including those without fluorescence properties. Similar to previously mentioned methods, they require specialized equipment and technical expertise and are costly, limiting their widespread use in research and clinical settings (Corica et al., 2022).

NMR is also capable but less utilized for detecting AGEs. It has advantages compared to LC, HPLC, UPLC and MS methods since it is capable of providing unambiguous detection of AGEs including detailed structural and quantitative information and quantitation in serum, plasma, urine, and other biofluids and tissues (João G. M. Pontes, 2017; Moises et al., 2023). Two recent discussions of AGE detection methods omitted use of NMR despite its complimentary attributes to other methods (Perrone et al., 2020; Takata et al., 2024). NMR may be less utilized for several reasons including the cost of NMR spectrometers, highly specialized training and limited accessibility compared to other methods.

Challenges and limitations detecting and quantifying AGEs include lack of standardized methods and reference materials (Poulsen et al., 2013; Smit & Lutgers, 2004). Comparison of results across studies and laboratories and species is challenging (Perrone et al., 2020; Poulsen et al., 2013). No method can simultaneously detect and quantify all types of AGEs, which may lead to potential underestimation or overestimation of total AGE levels when only one method is used

to estimate AGE levels in samples (Poulsen et al., 2013). Methods measuring fluorescent AGEs will miss non-fluorescent compounds while those targeting specific protein-bound AGEs will fail to measure free AGEs. The variability in sample preparation and the influence of factors such as diet, lifestyle, and underlying health conditions further complicate interpretation of AGE measurements. Total AGE pool is difficult to characterize since fluorescence techniques omit non-fluorescent AGE detection and blood based detection methods are not indicative of long term-presence or representative of tissue accumulation of AGEs (Perrone et al., 2020).

There is a need for more comprehensive studies to standardize measurement and establish reference ranges for AGE levels in healthy and diseased populations, which are currently lacking. AGE biomarkers are already used in clinical and research settings for humans, (e.g. for metabolic and renal disease), although current gaps in standardization and reference values continue to limit expansion of diagnostic and prognostic utility in specific disease states (Corica et al., 2022). Advancements for AGE detection have been recently validated, including monoclonal antibodies mapped with epitopes against CML with high sensitivity and high-throughput capability (Finco et al., 2016; Wendel et al., 2018). However, improvement for CML, and method establishment for other AGEs is necessary for wider adaption (Perrone et al., 2020; Wendel et al., 2018).

2.7.2 RAGE Detection & Quantification

Flow cytometry has been utilized to detect and quantify csRAGE expression in specific cell types (Sourris et al., 2010), RAGE distribution across immune cell types, and receptor regulation in varied physiological conditions (Akirav et al., 2012). Flow cytometry requires thorough technique optimization and validation of antibody specificity, staining protocols, and adequate controls (Cunliffe et al., 2009). Immunohistochemistry and immunofluorescence can analyze

RAGE distribution across tissues (Chen et al., 2020; Healey et al., 2019; Juranek et al., 2015) enabling assessment of receptor localization and expression patterns across cellular compartments. Although, like flow cytometry, western blot and ELISA, these methods face similar limitations including antibody specificity, sample preparation, and technique optimization.

RAGE measurement is also commonly achieved by ELISA, although other methods exist but with varying degrees of reliability. Like AGE detection, similar challenges exist regarding the accuracy of such testing due to the cross-reactivity of other proteins. This is evident in current RAGE testing methodologies, which specifically measure sRAGE and esRAGE. Since total sRAGE includes both cRAGE and esRAGE, subtraction of esRAGE results from total sRAGE results yields the cRAGE within the biosample (Prasad et al., 2016). Additional ELISA kits for other receptors interacting with RAGE are commercially available, although similar concerns exist regarding validation and antibody characterization. Limited species-specific antibody kits are also available.

Western blot analysis methods are also available for RAGE detection and have ability to distinguish between RAGE isoforms based on their molecular weight (Braley et al., 2016). This semi-quantitative method is useful for RAGE expression analysis, especially tissue specific distribution. However, like ELISA, western blot has limitations for antibody specificity and cross reactivity, limiting application and reliability.

Mass spectrometry-based (MS) methods are utilized for detection and quantification of RAGE. These methodologies offer high specificity and ability to identify post-translational modifications within samples (Holtz et al., 2021). Additionally, MS provides molecular mass and fragmentation patterns assisting in RAGE and associated isoform detection and quantification

(Moysa et al., 2019). However, sample preparation complexity, costs and need for specialized expertise limits widespread use. NMR is also capable of detecting and quantifying RAGE and isoforms, specifically in the context of protein concentrations and binding affinities of RAGE and its ligands (Koch et al., 2010). Although, it presents challenges for directly detecting and quantifying membrane-bound RAGE due to need for isotopic labeling (Koch et al., 2010; Manigrasso et al., 2016), potential interference of cellular membranes in flRAGE investigations as well as the low sensitivity of NMR, particularly for 15N experiments (Emwas et al., 2020). Regardless, NMR is valuable tool for determining structural information of RAGE and its isoforms, and has aided in defining structural RAGE details, domain structures, ligand binding sites and isoform characterization (Bongarzone et al., 2017; Koch et al., 2010; Kozlyuk et al., 2021).

2.7.3 Quantitative dAGE Databases

In 2015 the first quantitative database was published for the AGE concentration in 190 different food items utilizing UPLC-MS/MS (Scheijen et al., 2018). Until then, quantification of AGE in food products relied upon ELISA giving semi-quantitative results and had known issues with specificity and reproducibility. Previous AGE databases of foods were established with ELISA (Goldberg et al., 2004; Uribarri et al., 2010). This notable advancement using UPLC-MS/MS was made possible by the availability of AGE standards allowing for simultaneous detection and quantification of CML, CEL and MG-H1. Results showed distinct patterns in AGE distribution among differing categories of foods, with highest AGEs levels in peanuts, peanut butter, biscuits, cereals, toast and high-processed meats, among others (Scheijen et al., 2018). In contrast, the lowest AGE foods were coffee, fruits, vegetables, butter, olive oil, red wine and most cheese and milk products. Results contrasted earlier work that demonstrated high AGEs in butter, olive oil,

cheese and milk products and low levels in biscuits, crackers and cookies with ELISA (Uribarri et al., 2010). Given known limitations with ELISA, differences are explained by low specificity of antigen-antibody interactions and dependency on the chemical environment (Poulsen et al., 2013).

Additional AGE food databases have been published (Zhang et al., 2024). Database construction utilized UPLC-MS/MS and quantified 10 AGEs (CML, CEL, MG-H1, MG-H2, MG-H3, argpyrimidine, methylglyoxal-hydroimidazolone, glyoxal-hydroimidazolone, GOLD, and MOLD, with the most prevalent being CML, MG-H1, MG-H2, MG-H3, and G-H1) found in 334 foods within all major food groups in common Western and Chinese diets (Zhang et al., 2024). Foods highest in AGEs were processed nuts, bakery products, and some cereals and meats while low AGE foods were dairy products, vegetables, fruits and beverages.

There is currently no established AGE, MRP, or pet food processing database for pet food ingredients, finished formulations, or related products such as treats or supplements. Establishment of such a database would be a substantial advancement for research investigating dAGEs, and their accumulation in tissues and clearance mechanisms in dogs and other species.

2.8 Stability of AGEs

Examination of stability of AGE compounds in food, tissues and biofluids is underexplored. AGEs in food products are thought to be stable over time, although there is limited research confirming this assumption. Increases in AGEs result from the MR in thermal food processing such as grilling, baking, extrusion, or frying. It is thought AGEs including CML, CEL and MG-H1, remain relatively stable under elevated temperature conditions despite acceleration in precursor compounds such as 3-DG (Zhang et al., 2019). Stability was investigated in milk products processed conventionally compared to those processed with ultra-high temperatures and

subsequently stored at temperatures varying from 20-40°C for one year. Results showed milk processed at ultra-high temperatures had higher AGE amounts, and greater acceleration of precursor products compared to conventionally processed milk during storage. Curing and aging of dairy products may result in higher AGEs during storage over time (Sharma et al., 2015). Both results contrast assumptions on AGE stability in food products. It is possible that other factors could modify AGE levels in food including pH, fat content, sugar content, moisture and packaging although research is needed to investigate the impact of each of these factors over time.

Microbial activity within foods may degrade AGEs over time although this evidence largely appears to be extrapolated from studies on gut microbiota influence on AGEs. One study investigating *E. coli* in culture suggests some bacteria develop defense mechanisms to eliminate AGEs (Srebreva et al., 2009). Another study demonstrated ability of several human gut microbes, including *Klebsiella, Lactobacillus, and Escherichia-Shigella*, can enzymatically degrade protein-bound AGEs, hydrolyzing long-chain AGEs into shorter-chains (Shi et al., 2024). Others, like lactic acid bacteria, may prohibit AGE formation in foods (Li et al., 2022), however it is unknown if they have ability to degrade existing AGEs. Alternatively, other research has demonstrated *E. coli* can produce and secrete AGEs via energy-dependent efflux pump systems (Cohen-Or et al., 2011). In this instance, secreted AGEs accumulate extracellularly and have demonstrated to promote inflammatory responses in mammalian cells. Collectively, findings suggest microbial-derived AGEs play a role in host-pathogen interactions and systemic inflammation, although additional research is needed to better understand the complexities of these relationships.

In tissues, AGE stability is largely attributed to cross-linking of low turnover proteins (e.g. collagen, elastin) that are resistant to enzymatic degradation under physiological conditions. AGEs accumulate in the body with normal aging processes, associating them with aging and chronic diseases (Luevano-Contreras & Chapman-Novakofski, 2010). Circulating AGEs can be protein bound or free adducts. Free AGEs are subject to metabolic clearance while protein bound AGEs appear more stable and reflective of endogenous formation and accumulated dietary intake. Presence and stability of circulating AGEs is influenced by renal and liver function and associated disease. It is likely AGEs remain stable within tissues and biosamples under proper sample collection and storage, however there is limited data to confirm this. One study found plasma and serum CML and CEL were stable and the stability was not effected by common pre-analytical factors (e.g. collection tube type, delayed processing, storage temperature, and multiple freeze–thaw cycles) (Hull et al., 2013). Once excreted in urine, AGEs are also presumed to be stable under standardized sample handling and storage; however, this has not been investigated under varying storage conditions to the authors' knowledge.

2.9 AGEs & Chronic Disease: Human Evidence

A recent umbrella review of 16 meta-analyses identified consistent evidence linking ultra-processed (UP) food consumption to diabetes, cardiovascular disease incidence and mortality, colorectal cancer, and all-cause mortality (Barbaresko et al., 2024). While reliability for some studies is limited by study design and methodology, this review notes many chronic diseases are mechanistically linked to UP foods through dAGEs and other processing byproducts. This emphasizes broad impacts of UP foods on chronic disease development. Similarly, an umbrella review analyzing 45 pooled analyses from 14 meta-analyses with approximately 10 million participants found higher UP food consumption was associated with increased risks of mortality,

cardiovascular disease, type 2 diabetes, mental health disorders, and obesity (Lane et al., 2024). Specifically, individuals with greater UP food intake had 1.50-fold higher risk of cardiovascular disease-related mortality (95% CI 1.37-1.63, $p < 0.05$), 1.12-fold increased risk of type 2 diabetes per dose-response increment (95% CI 1.11-1.13, $p < 0.05$), and 1.55-fold higher risk of obesity (95% CI 1.36-1.77, $p < 0.05$) (Lane et al., 2024). Association to mental health was also observed, with odds ratios of 1.48 for anxiety (95% CI 1.37-1.59, $p < 0.05$) and 1.53 for combined mental disorders (95% CI 1.43-1.63, $p < 0.05$) (Lane et al., 2024). Findings highlight cumulative impact of UP food consumption on metabolic, cardiovascular, and neuropsychiatric health and strengthen concern UP diets, high in AGEs, contribute to global burden of non-communicable disease.

Another systematic review and meta-analysis including 13 randomized controlled trials also provides support that low-AGE diets positively impact health (Sohouli et al., 2021). Specifically, low-AGE diets significantly reduced insulin resistance, measured by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (mean difference: −1.204; 95% Confidence Interval [CI]: −2.057, −0.358; $p = 0.006$), and fasting insulin concentrations (−5.472 μU/mL; 95% CI: −9.718, −1.234; $p = 0.011$), compared to high-AGE diets (Sohouli et al., 2021). Results demonstrate dietary AGEs influence insulin resistance and importance of AGE reduction strategies to mitigate metabolic risk factors associated with diabetes and related disease including cardiovascular and renal disease.

AGEs contribute to cardiovascular disease and promote vascular dysfunction through two primary mechanisms. First, AGEs cross-link extracellular matrix proteins, leading to vascular stiffening. Second, they activate RAGE inducing oxidative stress and endothelial dysfunction (Hooshiar et al., 2022). These changes are also observed diabetes and hypertension. AGE/RAGE

activation exacerbates vascular injury by initiating cascade of events including upregulation of inflammatory cytokines, endothelial cell apoptosis, and increased expression of adhesion molecules further contributing to cardiovascular complications. Each of these mechanisms consequently impair vascular repair ultimately promoting atherogenesis (Pinto et al., 2022). AGE accumulation also exacerbates cholesterol uptake through macrophage activity, contributing to foam cell formation and atherosclerotic plaque development (Pinto et al., 2022). AGEs also disrupt reverse cholesterol transport by altering high density lipoprotein (HDL) structure and impairing receptor interactions, further exacerbating vascular injury and lipid dysregulation (Pinto et al., 2022).

AGEs are implicated in CKD through accumulation in renal tissues. Accumulation in the kidney, particularly in glomerular and tubular structures, activates RAGE signaling pathways that promote oxidative stress, proinflammatory cytokine release, mesangial expansion, and tubulointerstitial fibrosis. This establishes a continuing cycle, accelerating structural and functional decline in renal tissue due to glomerular hypertrophy and basement membrane thickening (Bohlender et al., 2005). Secretory RAGE (sRAGE), often proposed as a protective decoy, are reduced in CKD, potentially intensifying RAGE signaling (Molinari et al., 2021). Recent evidence shows when AGE clearance is impaired, they accumulate in renal tissues. Accumulation leads to AGE/RAGE interactions in renal epithelial and vascular cells, exacerbating cellular injury by increasing reactive oxygen species production and activating proinflammatory signaling cascades (Ma et al., 2025). Notably, these changes occur early in disease processes, before clinical signs of renal impairment, suggesting AGEs involvement in disease initiation and progression (Ma et al., 2025). High levels of circulating AGEs are associated with poor prognosis and increased mortality in CKD patients (Dozio et al., 2023).

Together, findings demonstrate the role of AGEs in cardiovascular and CKD, especially those with metabolic syndrome or diabetes. Although, elucidation of direct relationships between AGEs and these diseases is limited by challenges in nutrition research, especially isolating AGE effects among widespread UP consumption, limiting ability to understand and mitigate CKD progression.

2.10 AGEs in Dogs: Exposure & Emerging Evidence

Although AGE data in dogs is limited compared to humans, emerging studies document dietary exposure, systemic absorption, tissue accumulation, and possible inflammatory pathway modulation. As noted earlier, commercial pet foods, especially high thermal processed diets, may contain high concentrations of AGEs and MRPs, that vary by processing intensity. Given chronic consumption of UP diets (e.g. kibble and canned foods), cumulative effects of these compounds are likely.

Evidence from Palaseweenun et al. (2021) found dogs fed dry extruded kibble excreted higher levels of urinary AGEs, including CML, CEL, and LAL, compared to dogs fed raw diets (Palaseweenun et al., 2021). Median urinary CML excretion was 1,503 nmol/mmol creatinine in dogs fed dry food versus 603 nmol/mmol creatinine in raw fed dogs. Urinary AGE excretion present in raw fed dogs suggests endogenous formation and dietary absorption collectively contribute to total AGE burden (Palaseweenun et al., 2021). A separate study reported circulation plasma AGE levels did not vary by age, body size, or lifespan in domestic dogs, with similar patterns observed in wild canids (Jimenez, 2021). This suggests systemic AGE concentrations have tight physiological regulation, although long-term consequences remain unclear.

Evidence also suggests dietary processing level influences systemic AGE burden and AGE/RAGE dynamics in dogs. Bridglalsingh et al. (2024) evaluated differently processed diets;

UP wet (WF) and dry (DF), air-dried (ADF), and minimally processed cooked (MF) and found differences in plasma and urinary AGE concentrations (Bridglalsingh et al., 2024). Dogs fed UP diets (WF and DF) exhibited higher plasma total AGEs, including CML, CEL, methylglyoxal-H1, glyoxal hydroimidazolone-1, and argpyrimidine, compared to those fed less processed diets. Plasma total AGE concentrations were highest in WF-fed dogs (median 9.99 nM/50 μL) and lowest in MF-fed dogs (median 2.50 nM/50 μL), thus indicating dietary processing is associated with systemic AGE exposure (Bridglalsingh et al., 2024). The same study found higher urinary CML excretion in DF fed dogs compared to WF and MF groups. Higher CEL excretion was found in WF followed by DF, MF and ADF. Collectively this further supports dietary processing may alter AGE metabolism and excretion (Bridglalsingh et al., 2024). Although serum sRAGE concentrations did not significantly differ between diets, total AGE/sRAGE ratio was elevated in dogs fed UP diets with the ratio higher in MF vs. WF ($p = 0.018$) and ADF vs. DF ($p = 0.036$). AGE/sRAGE ratio marks systemic AGE burden relative to receptor buffering capacity, thus suggesting higher dietary AGE intake results in ratio shifts. These shifts have been previously associated with inflammation and oxidative stress in other species (Cepas et al., 2020; Luevano-Contreras & Chapman-Novakofski, 2010). Although the study did not establish causality or direct disease outcomes, it provides initial evidence that dietary processing level affects systemic AGE load and receptor dynamics in dogs, when nutrient targets are formulated to meet similar nutrient profiles (Bridglalsingh et al., 2024).

Studies examining tissue accumulation of AGEs provides further support. Shapiro et al. (2008) demonstrated AGEs accumulate in vascular smooth muscle of dogs with atherosclerosis and results in modification of vascular, but not valvular, structure (Shapiro et al., 2008). Chiers et al. (2010) reported AGE accumulation in arterial plaques of dogs, noting correlation between

AGE presence and inflammatory cell infiltration (Chiers et al., 2010). Previously, AGE accumulation was detected in cerebellar neurons of aged dogs with localization patterns similar to human Alzheimer's disease, suggesting potential role in cognitive decline and associated cognitive degenerative diseases (Weber et al., 1998). Although these studies do not establish causal relationships between dietary AGE exposure and disease, they provide evidence AGE tissue accumulation is relevant to age-related disease.

RAGE pathway dysregulation has been observed in dogs with chronic GI disease. Heilmann et al. (2014) reported dogs with inflammatory bowel disease (IBD) had lower serum soluble RAGE (sRAGE) concentration compared to healthy dogs (Heilmann et al., 2014). Median sRAGE concentrations were 381 pg/mL in dogs with IBD compared to 1,024 pg/mL in healthy dogs ($p = 0.001$) (Heilmann et al., 2014). Notably, sRAGE has been suggested as a biomarker of disease risk and adverse outcomes, functioning as a decoy receptor by binding AGEs and preventing activation of membrane-bound RAGE (Erusalimsky, 2021). In the Heilmann study, dogs achieving complete remission following treatment exhibited increased sRAGE concentrations, suggesting sRAGE restoration may play a role in resolving inflammation (Heilmann et al., 2014). Cabrera-García et al. (2021) found dogs with chronic inflammatory enteropathy exhibited significantly higher RAGE expression in duodenum and colon tissues compared to healthy controls (A. I. Cabrera-García et al., 2021). Specifically, RAGE expression was elevated in apical and basal regions of both intestinal segments (duodenum apical: 10.36 vs. 6.75 ($p = 0.0152$); duodenum basal: 12.72 vs. 6.09 ($p = 0.0033$); colon apical: 10.24 vs. 6.32 ($p = 0.0029$); colon basal: 11.64 vs. 7.65 ($p = 0.0359$)) (A. I. Cabrera-García et al., 2021). The study also demonstrated positive correlation between serum sRAGE and duodenal RAGE expression, indicating possible compensatory mechanism or dysregulation of sRAGE-RAGE axis in chronic

GI inflammation. The study confirmed *in vitro* S100/calgranulin proteins as ligands for RAGE in dogs, demonstrating pathway involvement in chronic GI inflammation (A. I. Cabrera-García et al., 2021). While these studies do not directly link dietary AGEs to GI disease in dogs, they collectively provide evidence of dysregulation in RAGE signaling pathways in chronic inflammation.

High thermal processed diets may increase inflammatory pathway activity in dogs. Dogs fed these diets, compared to those fed raw diets, showed elevated metabolites associated with sulfur amino acid metabolism and inflammation, including increased oxidative stress markers (Moore et al., 2020). Although MRPs were not directly measured, some metabolite patterns align with pathways influenced by dietary AGEs in other species (Tuttle et al., 2005), possibly indicating MRP-related oxidative stress may be a contributor. Authors of the same study found metabolite changes associated with gut microbial metabolism and redox status (Moore et al., 2020), providing additional support for dietary processing influencing host metabolism and microbiome-mediated pathways. Recent evidence also provides evidence of dietary modulation of AGE burden. Another study demonstrated dietary supplementation with soluble fiber and omega-3 fatty acids reduced circulating AGEs and uremic toxins in senior dogs (Ephraim et al., 2020). Supplementation was associated with gut microbial composition changes, including increased butyrate-producing bacteria and reduced taxa associated with inflammation (Ephraim et al., 2020). These studies suggest dietary interventions influence AGE metabolism and modulate inflammatory pathways, providing potential strategy for mitigating AGE-risk.

Another study, while not measuring AGEs directly, compared effects of differently processed diets and directly supports use of processing intensity as a primary classification criterion. The randomized cross-over study demonstrated healthy dogs fed a cooked whole food

diet exhibited shifts toward anti-inflammatory immune profiles compared to when the same dogs consumed a UP dry food (Jaffey et al., 2022). Lipoteichoic acid (LTA) stimulated leukocytes produced lower tumor necrosis factor-alpha (TNF-α) to interleukin-10 (IL-10) ratio in dogs fed a whole food diet compared to when fed UP diet (Jaffey et al., 2022). Findings indicate suppression of systemic pro-inflammatory signaling in dogs fed lesser processed diets. The same dogs exhibited elevated interleukin-8 (IL-8) production in LTA-stimulated leukocytes, suggestive of increased neutrophil activity and innate immune responsiveness even in absence of increased baseline inflammation. In addition, acute phase proteins (serum amyloid A, C-reactive protein, haptoglobin), oxidative burst capacity, and baseline cytokine production were assessed. No significant differences were observed between diets for these markers, suggesting dietary intervention influenced immune responsiveness rather than basal systemic inflammation (Jaffey et al., 2022). Findings demonstrate processing intensity and diet composition may shift immune system response, potentially influencing disease resilience and response to immune triggers, even in absence of overt inflammation (Jaffey et al., 2022).

These immune modulating effects of diet processing may have implications for inflammatory and allergic conditions. Given atopic dermatitis and other allergic conditions are increasingly common in dogs (Zhak et al., 2024), dietary processing byproducts may play roles contributing to immune dysfunction. The complexity of atopic dermatitis and the unclear role of diet in its pathogenesis are well recognized (Rustad et al., 2022). Current veterinary guidelines primarily recommend high thermally processed diets, such as hydrolyzed or therapeutic formulations, for the management of allergic and atopic conditions in dogs; however, these recommendations frequently overlook the potential immunologic effects of processing byproducts. Hydrolyzed protein diets, enzymatically broken down to reduce antigenicity,

undergo multiple rounds of high-thermal processing, likely increasing AGE and histamine formation. Consequently, it is possible this could inadvertently increase systemic burden in some dogs, although remains unproven. For example, hydrolysis generates free amino acids and small peptides, including lysine and arginine residues, highly susceptible to MR during thermal processing, used to manufacture hydrolyzed diets (De Almeida, 2013; Oliveira et al., 2021). It is possible this increases formation of reactive MRPs in the final product. Second, ingestion of hydrolyzed proteins may increase pool of free amino acids available for endogenous AGE formation *in vivo* (Chaudhuri et al., 2018; Twarda-Clapa et al., 2022). This is especially true under conditions of oxidative stress or poor glycemic control, both common in chronic inflammatory diseases (Blanca et al., 2024).

Furthermore, cumulative effects of hydrolysis combined with multiple rounds of high-thermal processing likely increase overall burden of processing byproducts consumed (van Rooijen et al., 2013) since use of industrial ingredients, like rendered meat meals, plant protein isolates, and starch concentrates, are subjected to various chemical and thermal treatments prior to final extrusion or canning. Given AGE involvement in receptor-mediated inflammatory pathways, and immune dysregulation in other species (Chaudhuri et al., 2018; Twarda-Clapa et al., 2022), potential role in allergic disease in dogs fed hydrolyzed UP diets merits investigation. To the author's knowledge, no published studies exist quantifying MRP concentrations in hydrolyzed veterinary therapeutic diets, despite widespread use for canine atopic conditions.

Recent research provides additional context from a dietary macronutrient rather than processing perspective. A controlled study of ten clinically healthy dogs compared physiological responses to two fresh food diets: a control diet (high-fiber, low-fat) and Western-style diet (high-fat, high-carbohydrate) across three sequential one-month feeding periods (Mason et al.,

2025). Findings demonstrated Western diet consumption was associated with elevated serum high-sensitivity C-reactive protein levels and increased colon NF-κB expression suggesting increased systemic inflammation (Mason et al., 2025). Histopathological examination of colonic biopsies revealed mild to moderate inflammatory lesions, including mucosal edema and crypt distortion, providing morphological evidence of diet-induced intestinal damage (Mason et al., 2025). These structural changes may compromise intestinal barrier function, potentially facilitating increased systemic exposure to dietary AGEs and other MRPs present in thermally processed foods. Microbiota disruption was also noted, characterized by increased total bacterial load and shifts toward bacteria associated with adverse health outcomes (Mason et al., 2025). This dysbiosis occurred alongside bile acid metabolism disturbances, notably increased fecal cholic acid concentrations. Such alterations may create an intestinal environment enhancing AGE formation and absorption since bile acids influence inflammatory pathways and oxidative stress mechanisms implicated in AGE-related pathologies. These inflammatory markers are similar to patterns observed in AGE-induced pathological states in humans (Twarda-Clapa et al., 2022), suggesting potential mechanistic overlap between thermal processing effects and macronutrient-driven inflammation in dogs.

Another study focused on macronutrient composition rather than processing techniques and found elevated inflammatory markers (NF-κB and C-reactive protein) similar to those in AGE-related conditions (García-García et al., 2021; Zhong et al., 2015). Further, results may suggest interaction between macronutrient composition and food processing contribute to endogenous AGE formation and subsequent health impacts. Since physiological changes observed mirror those in human research, it also demonstrates challenges in isolating dietary variables since changes in macronutrient content inherently affect food preparation methods, also

potentially altering AGE content. This complexity demonstrates why researchers and veterinarians must consider both dietary composition and processing methods to assess individual and combined health effects in their respective settings. These findings support development of comprehensive classification systems integrating macronutrient profiles and processing techniques for understanding food quality implications on companion animal health. Optimizing macronutrient balances to mitigate high-fat and high-carbohydrate diets may diminish risk of AGE-associated chronic diseases in clinical and commercial feeding settings.

Despite evidence of exposure, absorption, excretion, tissue accumulation, and metabolic pathway involvement, several gaps remain in understanding the long-term effects of chronic AGE exposure in dogs. Critics of potential risks posed by high thermal processed foods in dogs note distinct differences between human UP food and high thermally processed foods. Unlike human foods, often devoid of essential nutrients, these pet foods are formulated to be nutrient-rich to meet physiologic needs of pets (Sanderson, 2021; Witzel Rollins, 2024). High thermally processed pet foods typically contain carbohydrates derived from cereal grains or alternatives, either whole or fractionated, and may comprise up to 50 percent of the total formulation (Beloshapka et al., 2016; Bradshaw, 2006). These carbohydrates are composed primarily of complex, rather than simple, sugars, in comparison to many UP human foods which have an excess simple sugar content. Additionally, sodium is moderate compared to human UP foods, although it has been noted that many commercial formulations for dogs exceed recommended sodium levels (Beynen, 2017). Proponents of these pet foods claim there are no studies conclusively proving fresh diets are superior to commercial diets. They further assert the pet food industry follows regulatory standards ensuring quality and safety, all of which moderate risks (Sanderson, 2021; Witzel Rollins, 2024).

No studies have established direct causal relationships between dAGE intake and chronic diseases such as obesity, diabetes mellitus, chronic kidney disease, or cardiovascular disease in dogs. Studies evaluating whether sustained dAGE exposure alters inflammatory pathways, oxidative stress markers, or metabolic outcomes are lacking compared to humans. Further, interaction between dAGE intake, gut microbiota, and systemic inflammation in dogs has not been explored in detail, despite emerging evidence from human studies suggesting such interactions play roles in diseases affecting humans and dogs (Barbaresko et al., 2024; Bridglalsingh, 2020; Lane et al., 2024; Oba et al., 2022).

Comparative work suggests dogs may exhibit species-specific differences in AGE metabolism, and highlights need for species specific research. For example, serum albumin in dogs reportedly has lower susceptibility to glycation compared to humans and potentially indicates differences in protein protection mechanisms and other pathways (Rivera-Velez et al., 2019). Nevertheless, dogs' chronic exposure to UP diets may overwhelm these protective mechanisms over time, particularly when antioxidant and phytochemical intake is low.

Collectively, the studies provide evidence dogs are exposed to dAGEs, absorb and excrete them systemically, and exhibit changes in regulatory pathways; however, gaps remain in understanding how chronic exposure to UP food influences health outcomes in dogs. Given rising prevalence of chronic inflammatory and metabolic diseases, investigating roles of dAGEs and other processing-derived compounds represents an area of research priority in veterinary medicine. Addressing these gaps is needed for developing dietary recommendations and evidence-based classification systems evaluating pet foods based on processing level, nutrient adequacy and other related factors.

2.10.1 AGE Content of Commercial Pet Foods

Recent investigations have expanded understanding of dAGE consumption by dogs. Fructoselysine (FL), CML, hydroxymethylfurfural (HMF), and lysinoalanine (LAL) were measured in 67 commercial dog and cat foods and found to be in highest concentration in canned foods, followed by pelleted and extruded dry foods (van Rooijen et al., 2014). MRP levels varied between types of processing, suggesting pets consuming these foods may ingest considerably higher amounts of MRPs on a metabolic bodyweight basis compared to humans eating a Western diet that is ultra processed and typically high in AGEs. A separate comprehensive review of MR in pet food associated with high thermal processing, emphasized the impact on dietary quality and potential health implications for pets (van Rooijen et al., 2013). MR reduced the bioavailability of essential amino acids, particularly lysine by up to 61.8%, resulting in decreased dietary protein bioavailability and negatively impacting nutritional adequacy especially for growing dogs.

In another study, feeding four differently processed diets having similar nutritional profiles to healthy dogs showed that canned wet foods contained the highest dAGEs (Bridglalsingh et al., 2024). High levels of AGEs in canned diets correlated with elevated plasma AGE levels compared to other diets. It is unknown if this would be the same in dogs eating such diets over a long period due to lack of data. Canned foods typically containing higher amounts of MRPs may seem counterintuitive since thermal processing preserves moisture compared to extrusion or pelleting with dry, kibble diets, which has been reported to result in lower AGEs. Canned foods have a longer cooking time compared to extruded diets, which could contribute to higher AGEs.

Estimating total AGE content by measuring one or a few AGEs is inaccurate due to variability in AGE formation. Van Rooijen et al. reported higher FL, CML, and HMF levels in canned diets on a dry matter basis, but when expressed as-fed, canned diets showed lower CML and HMF, while dry diets had the highest HMF (van Rooijen et al., 2014). This highlights how moisture content impacts interpretation. Neither as-fed nor dry matter accounts for actual dietary exposure. A per-calorie basis better reflects AGE intake by normalizing for moisture and energy consumption. Without standardized methods for reporting AGE content in food, selective measurement of individual AGEs can misrepresent total AGE load and dietary exposure. This is especially problematic given the variability in AGE profiles across food types and processing methods. These gaps highlight necessity for consistent detection, quantification, and reporting protocols for AGEs in food matrices. Without such standardization, accurately characterizing dietary AGE exposure and associations to health outcomes will remain challenging.

2.11 Differences Between Human & Canine Metabolism

Dogs have different digestive and metabolic responses to various nutrients compared to humans and it is important to consider when evaluating health impacts of dietary components and the nutritional matrix of diets. Healthy dogs may tolerate higher dietary fat and protein in absence of caloric excess since they are efficient at deriving energy from these macronutrients (Hand et al., 2010). However, extensive processing of proteins and fats in UP foods can decrease digestibility and bioavailability leading to the formation of AGEs and other potentially detrimental compounds (van Rooijen, 2015). Humans benefit from dietary fiber that is fermented by gut bacteria producing SCFAs supporting gut health. The dogs' gut microbiota and fermentation processes differ with certain fiber types having potentially different impacts on gut health and nutrient absorption ((NRC), 2006).

While some sources classify dogs as omnivores (Fascetti & Delaney, 2023), the Nutrient Requirements of Dogs and Cats by the National Research Council ((NRC)) states dogs are carnivores ((NRC), 2006). More recent definitions characterize dogs as facultative carnivores due to their adaptive capacity to digest starch, supported by increased pancreatic α-amylase activity (He et al., 2024). This enzymatic adaptation allows dogs to utilize starch-rich diets more efficiently than obligate carnivores like cats, supporting their survival alongside humans through dietary adaptation. Nevertheless, UP commercial diets are often formulated with substantial carbohydrate content for numerous reasons including cost, shelf stability, and improved physical structure of kibble or canned products. These carbohydrate rich formulations contribute a large portion of dietary energy. While increased carbohydrate intake theoretically raises circulating glucose and enhances substrate availability for endogenous glycation, healthy dogs appear to have lower susceptibility to protein glycation under hyperglycemic conditions compared to humans (Rivera-Velez et al., 2019). This is potentially due to species specific control mechanisms, but this does not preclude long term risk. Chronic exposure to carbohydrate-rich, UP diets may still promote endogenous glycation or AGE accumulation through alternative pathways, particularly in aging or metabolically stressed dogs.

Additionally, thermal processing reduces the bioavailability of essential amino acids like lysine and arginine through degradation or binding reactions. This combination of processing losses and formulation choices helps explain why lysine, arginine, and other essential amino acids are often supplemented in UP pet foods to meet nutritional adequacy despite potential glycation-related risks.

2.12 Challenges in Nutrition Research

There are major challenges in nutrition research, including those investigating dietary impacts on health and disease. One of these challenges in human nutrition research is lack of true control groups, as UP foods account for approximately 58% of total daily caloric intake among U.S. adults and 67% among children and adolescents (Juul et al., 2021). Widespread UP food consumption makes it difficult to assess health outcomes in individuals with minimal exposure. Additionally, distinguishing direct effects of UP foods from secondary risk factors like obesity is also challenging. In the Framingham Offspring Study, each additional daily serving of UP foods was associated with 7% increased risk of hard cardiovascular disease (CVD), 9% increased risk of coronary heart disease (CHD), 5% increased risk of overall CVD, and 9% increased risk of CVD mortality, after adjusting for total energy intake, diet quality, waist circumference, and body mass index (BMI) (Juul et al., 2021). Findings suggest that while obesity is a major risk factor for cardiometabolic disease, UP foods likely have independent effects that extend beyond obesity.

Although studies discussed here demonstrate association between UP food consumption and increased risk of these and other disease, several limitations exist. Many studies included reported modest effect sizes, (e.g. relative risks ranging from 1.1- to 1.5-fold increases) which may not be meaningful based on disease and other factors. However, a 1.5-fold increase in cardiovascular disease, for example, remains clinically meaningful. Other challenges in human nutrition research and studies discussed in this section include detection of tiny effect sizes among confounding variables, inaccuracies in dietary measurement (Gemming et al., 2014), and inadequacies of small, short-term studies that lack clear, actionable results (Trepanowski & Ioannidis, 2018). Given these challenges, dogs are a valuable comparative model for studying

long-term dietary exposures in a more controlled, yet realistic setting. Unlike human populations with varied dietary habits, dogs are typically fed consistent diets reducing confounding variables and thus offer a promising solution to challenges inherent in human nutrition research. Owners who choose minimally processed diets may be more engaged in their pets' health and nutrition, making them also likely to adhere to strict feeding protocols for minimally processed diets. While formal studies on compliance among these pet owners are limited, this is a potential unique opportunity for research. Controlled trials in dogs could help bridge gaps in human nutrition research, offering insights into dietary AGEs, processing byproducts, and long-term metabolic health outcomes that are difficult to achieve in human populations. Although evidence presented here focuses on human health, mechanisms provide context for understanding how AGEs, may similarly impact companion animal health. Given dogs are often fed the same processed diet for extended periods, typically a lifetime, cumulative exposure to AGEs likely exceeds that of humans. Moreover, molecular pathways involved in AGE accumulation and RAGE-mediated inflammation are conserved across species (Murua Escobar et al., 2006), supporting likelihood that dogs share similar vulnerabilities to chronic dietary AGE exposure.

2.13 Dogs as Translational Models for Nutrition Research

Dogs share many metabolic and inflammatory diseases with humans, including obesity, diabetes mellitus, chronic kidney disease, osteoarthritis, and cancer (Hoffman et al., 2018). These diseases are linked to chronic inflammation, oxidative stress, and metabolic dysfunction, mechanisms also driven by dAGEs and other MRPs (Uribarri et al., 2010; Poulsen et al., 2013). Dogs share environments, lifestyles, and spontaneous development of chronic diseases common in humans, including cancer, diabetes, and inflammatory conditions. Additionally, their structured breeding results in genetic uniformity within breeds and diversity across breeds. In theory, this can

somewhat simplify and streamline identification of disease-associated genes, providing translational value for shared conditions, especially those exacerbated by dietary factors like AGEs and associated receptor interaction. Further, dogs have closer genomic similarity to humans than rodent models, and provide a practical and efficient models for investigating diet-related health outcomes and chronic disease susceptibility (Shearin & Ostrander, 2010). Nevertheless, despite growing evidence connecting UP diets to chronic disease in humans, veterinary nutrition has yet to systematically evaluate impact of similar processing byproducts in these diseases in dogs limiting benefit to both species.

Dogs typically eat solely or predominantly thermally processed commercial dry diets which provides an opportunity for longitudinal assessments of dietary exposure and health outcomes. Commercial thermally processed pet foods like kibble and canned diets are intended to be complete and balanced mainstays of dogs diets, with similarities in formulation, palatability, processing, packaging, shelf-life and characteristics to that of UP human diets. Unlike rodent models, which require artificial induction of disease, dogs naturally develop chronic diseases in patterns like humans. This makes them ideal comparative biomedical models and offers an opportunity to bridge gaps between laboratory-based research and dietary exposures in humans.

Dogs' shorter lifespans also provide practical advantages allowing for observation of dietary impact on disease progression and outcomes within condensed timeframes. Despite advantages, veterinary research has focused on short-term feeding trials designed to meet nutrient minimums, over assessment of effects of chronic ultra-processed diet and associated MRP exposure. Trials generally are limited to 26 weeks for adult maintenance under AAFCO guidelines ((AAFCO), 2025), and insufficient for evaluating long-term metabolic and potential

inflammatory consequences of commercial thermally processed diets. Furthermore, research on commercial pet foods is largely funded by industry stakeholders (Phillips-Donaldson, 2023), it is plausible that this limits scope and may provide reasoning for excluding evaluations of processing methods, such as MRP formation.

Evidence supports dogs are chronically exposed to high levels of dAGEs and other byproducts, exceeding human exposures on a metabolic body weight basis (van Rooijen et al., 2014; Bridglalsingh et al., 2024); however physiological exposure consequences remain poorly studied. Given AGEs are linked to oxidative stress, systemic inflammation, metabolic dysfunction, and insulin resistance in humans (Chaudhuri et al., 2018; Dozio et al., 2023), similar pathways are likely in dogs, warranting further investigation. Despite clear opportunities, regulatory agencies lack guidance for AGEs or other MRPs in pet foods, leaving gaps in dietary safety standards. Integrating a processing-based classification system, alongside research into long-term exposure effects, would facilitate better regulation, improved product transparency, and safer dietary options for companion animals. Such advancements would also support cross-species insights into how industrial food processing shapes chronic disease risks, benefitting both veterinary and human medicine.

2.14 Knowledge Gaps & Future Directions

Despite decades of research, reliable detection, quantification, and isolation of all AGEs has not been realized. A major challenge is the broad nature of AGE physical properties, molecular polarity, molecular weights, and fluorescence. This heterogeneity means no singular detection method can detect all known AGEs, which is a limiting factor for AGE-related investigation.

Stability of AGEs within biological samples such as plasma and urine are underexplored in veterinary medicine. These gaps limit analyses involving AGEs and receptor interactions

within the various biological compartments. These limitations highlight necessity for standardized methodologies and quality control practices to better integrate AGE research into food processing investigations, safety, and health. Additionally, substantial gaps exist within the literature regarding standardization for sample handling for dog metabolomics research. This limitation also limits advancement of metabolomics research within veterinary medicine and thus discovery of reliable biomarkers, including AGEs, for disease detection and monitoring. While human metabolomics literature documents methodology and demonstrates clear relationships between handling protocols, storage conditions, and sample stability, veterinary studies, particularly canine metabolomics, often have methodological inconsistencies. These include sample storage delays and improper storage conditions that may compromise results since metabolites are susceptible to chemical, enzymatic and bacterial factors.

While establishment and utilization of human food AGE databases is becoming more common, there is currently a lack of a formally established database for pet food ingredients, finished formulations and related products such as treats or supplements. Establishment of such database would be meaningful for AGE investigations regarding intake and clearance mechanisms in dogs and other species. Pet food categorization like human food has not been fully realized or accepted to date, despite a proposal and growing evidence suggesting processing methods influence on nutrition quality and health outcomes. This is yet another area in need of attention in veterinary nutrition since dogs commonly consume commercial pet foods comprised of ingredients subjected to extensive thermal processing.

Advancement in areas explored within this chapter will be dependent upon standardized detection methodologies and clarification of AGEs metabolic mechanisms. Establishment of standardized protocols, expansion of spectral databases, and development of comprehensive pet

food databases will substantially aid in advancing understanding of AGE metabolism, accumulation and health consequences across species.

2.15 Purpose of This Research

This purpose of this research was to identify stability and viability of dog urine under different time and temperature conditions for use in metabolomics studies. This information will provide recommendations for sample handling and storage recommendations for the veterinary field and influence future study design. Stability of AGEs in urine over time is also undocumented, presenting known and unknown barriers to research involving these compounds. This research investigated stability of CML and CEL over time at room temperature to understand the viability of urine collected for related investigations. This knowledge is necessary for establishing evidence-based protocols for sample handling in veterinary and human metabolomics research, including AGEs.

Collectively, this research addresses gaps in methodology and urine stability that limit progress in both veterinary and human medicine. By standardizing urine metabolomics protocols and characterizing AGE stability in biological matrices this work establishes foundation to advance comparative research. These contributions enable targeted investigations of AGEs in health and disease, support future studies on diet, aging, and metabolic disorders across species. The comparative approach leverages the dog as an efficient model for dietary research, with outcomes informing both veterinary practice and human medicine.

CHAPTER 3

ASSESSMENT OF SAMPLE HANDLING CONSISTENCY AND REPORTING IN DOG URINE METABOLOMICS STUDIES[1]

[1]Nicole R. Cammack, Joseph W. Bartges. To be submitted to a peer-reviewed journal.

Abstract

Metabolomics is increasingly applied in veterinary research for investigation of disease mechanisms, biomarker identification, and nutrition. Urine is a valuable biofluid for such studies due to its non-invasive collection and rich metabolite profile. To evaluate methodological reporting of available studies, we reviewed 25 peer-reviewed dog urine metabolomics studies published between 2007 and 2024. Analysis revealed variable reporting practices, with methodological details often absent or insufficiently described for replication. Collection methods, handling procedures, and preparation steps were frequently underreported, and temperature control before and during data acquisition was inconsistently addressed. These findings highlight the opportunity to improve standardization and reproducibility in veterinary metabolomics research. Adoption of clear, consistent reporting practices aligned with established human metabolomics protocols, adapted for veterinary applications, would enhance data quality, facilitate cross-study comparisons, and support the growing utility of canine urine metabolomics in veterinary medicine, nutrition, and comparative research.

3.1 Introduction

Metabolomics is used for investigating disease mechanisms and developing diagnostic capabilities in dogs. As part of broader 'omics' technologies, metabolomics provides insights into dynamic physiological states advancing understanding of health and disease across species (Clish, 2015; Stevens et al., 2019; A. Zhang et al., 2012). While metabolomics has been introduced into human clinical practice, veterinary applications remain limited despite research potential (Graciela et al., 2020). Dogs are valuable comparative models due to shared environments, lifestyles, and chronic diseases that parallel humans including diabetes mellitus, obesity, arthritis, cancer, chronic kidney disease, and cognitive dysfunction. The shared environmental exposures and consumption of largely ultra-processed diets (van Rooijen et al., 2014) with dogs' accelerated aging provide opportunities for longitudinal and comparative studies. Dogs provide potential for insights into disease etiopathogenesis, diagnosis, progression and management that are applicable to human and veterinary medicine and nutritional research.

Urine provides a biofluid that can be collected non-invasively and used for metabolomics due to its abundant metabolites (Bouatra et al., 2013). Human metabolomics literature describes protocols for sample collection, handling, storage, and preparation that aid in reproducibility and reliability of results (Abdul-Hamid Emwas, 2016; Anthony C. Dona, 2014; Emwas et al., 2015; Lauridsen et al., 2007; Michael et al., 2007; Rainer Lehmann, 2020; Raúl et al., 2020; Richard et al., 2008; Rist et al., 2013; Rotter et al., 2016). These standardizations are critical since metabolites in biological samples like blood, urine, and tissues are sensitive to chemical, enzymatic, and bacterial factors, especially during storage delays and temperature fluctuations. Human studies document relationships between handling protocols, storage conditions and sample stability (Abdul-Hamid et al., 2015; Raúl González-Domínguez, 2020). However,

veterinary medicine lacks comparable standardized protocols for dog urine collection and processing and storage methods for metabolomics research.

Published canine urine metabolomics studies demonstrate methodological inconsistencies when compared to established human protocols. These variations limit interpretation of results particularly for disease characterization, biomarker identification, and development of diagnostic or therapeutic targets. Non-standardized pre-analytical steps may introduce unintended yet critical sources of variability between samples and subsequent analyses (Raúl et al., 2020). Therefore, there is need for standardized protocols regarding dog urine collection, handling, and storage for metabolomics research to improve reproducibility and advancement of the field.

3.2 Methods

We reviewed dog urine metabolomics publications evaluating the consistency and quality of sample collection, handling, storage, and preparation methods and documentation. Articles included original research on dog urine metabolome in breed, aging, health, disease, or nutrition, using techniques like mass spectrometry (MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC). Pubmed, Science Direct, and Web of Science were initially searched using terms (dog or canine or Canis lupus familiaris) and (urine) and (metabolomics or metabolomic or metabonomics or metabonomic or metabolome). Subsequent query in the same databases included terms (dog or canine or Canis lupus familiaris) and (urine) and (metabonomics or metabonomic or metabolic profiling) not (rodent or human). Both searches were from database inception to May 5, 2024. Studies were excluded if they met any of the following criteria: conference abstracts or proceedings, book chapters, review articles, non-English publications, and drug efficacy or toxicity studies (Figure 3.1) (Page et al., 2021).

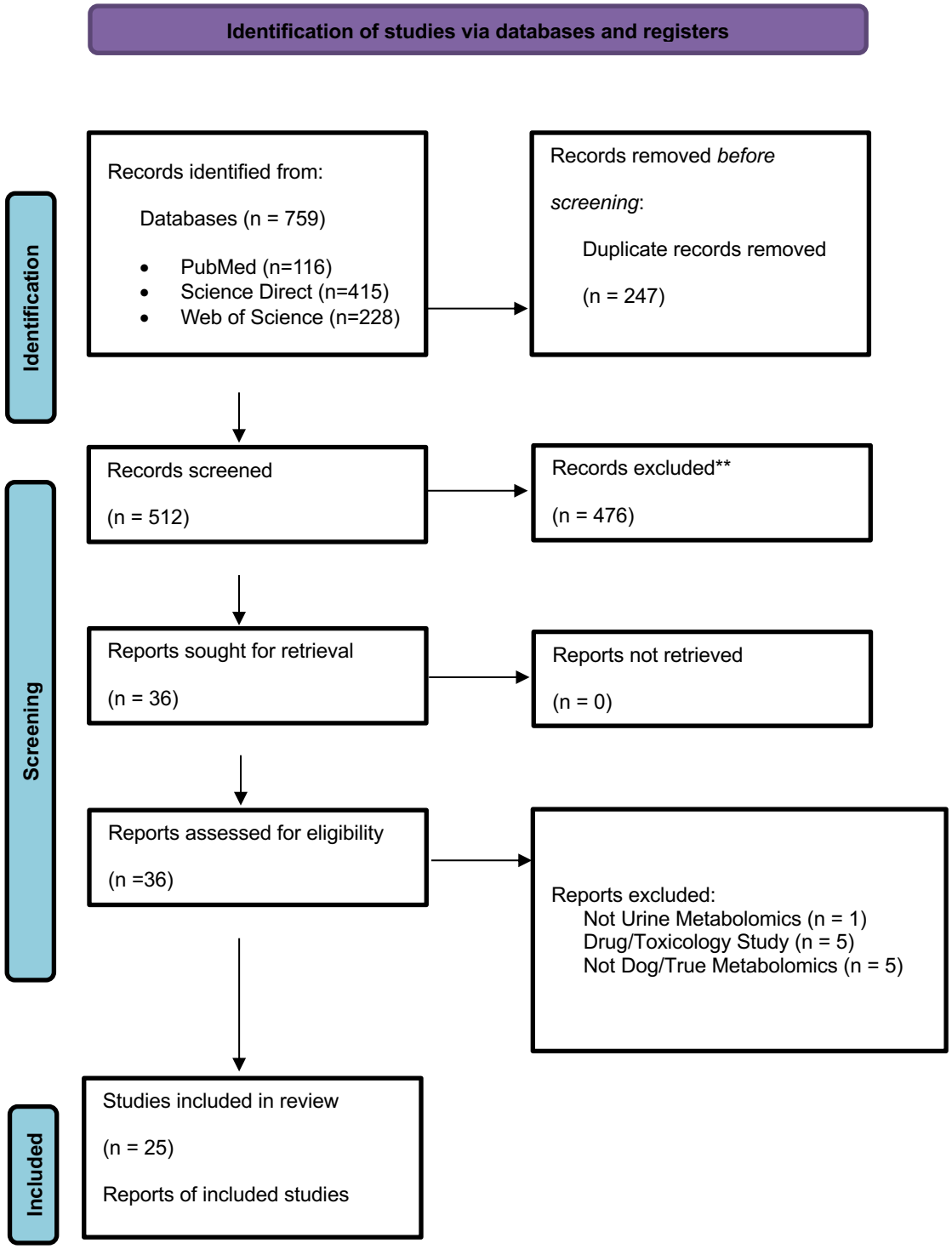

Figure 3.1 PRISMA flow diagram of study design

Each publication was assessed using a predefined checklist of reporting criteria related to sample collection, handling, storage, and preparation methods (See Table 3.1 & Appendix A). For each item, a binary response ('Yes' or 'No') was recorded based on whether the information provided was sufficient to permit replication. The approach was not intended to serve as a quantitative scoring system, but rather a qualitative audit of methodological transparency across studies (See Appendix A). In cases where methodological details were implied but not explicitly stated, a conservative approach was taken; responses were recorded as "No" unless the information was clearly described and replicable without inference.

Table 3.1 Data included of Dog Urine Metabolomics Studies.

Metabolomics Platform
Was the sample collection procedure clearly defined?
Was the sample collection container provided?
Was the sample collection method the same for each subject?
Was the sample storage preparation clearly defined?
Was the aliquot amount, sample storage container/type/manufacturer provided?
Was the sample storage condition clearly defined?
Sample Storage Condition Temperature
Was the time between collection and storage clearly defined?
Was the sample thawing method clearly defined?
Was the sample preparation for data acquisition clearly defined?
Was the amount of reagent(s) manufacturer/ source provided?
Was the manufacturer/source for sample preparation containers and data acquisition tubes provided?
Were the samples temperature controlled prior to data capture?
Were samples temperature controlled during data acquisition?

3.3 Results

Initial database searches returned 759 results, with 25 studies published between 2007 and 2024 remaining after duplicate removal and excluding non-dog studies and studies not meeting additional inclusion criteria (Table 3.2). Of the 25 included studies, 16 (64%) were published between 2020-2024. Mass-spectrometry was used in 17 (68%) of studies, 6 (24%) used NMR, and 2 (8%) used UPLC or HPLC.

Analysis of methodological reporting revealed variability and gaps in the following: sample collection, sample handling, sample storage, sample preparation, and temperature control.

3.3.2 Sample Collection

Of 25 studies, three (12%) clearly defined urine collection method(s) (e.g., free-catch, cystocentesis) and collection container. The remaining 22 studies (88%) lacked clear definitions of procedures and/or failed to specify collection container type and manufacturer. Furthermore, 11 of 25 studies (44%) employed consistent collection methods across all subjects, while 14 of 25 studies (56%) used mixed collection methods (e.g. combination of free catch, cystocentesis and/or catheterization) or did not report methodology (Table 3.3). Collection container specifications were reported in 3 of 25 studies (12%) with 1 study (4%) comprehensively detailing both storage container and aliquot amount.

3.3.3 Sample Handling

Of the 25 studies, 21 (84%) either omitted procedures entirely or provided insufficient detail for replication, while 4 of 25 studies (16%) reported methods adequately for repeatability. Reported handling procedures showed considerable variation, including different combinations of centrifugation, filtering, antimicrobial addition, and chilling protocols.

Table 3.2 Included dog urine metabolomics studies published between 2007-2024.

Year	Title	Authors	doi
2007	Validation of a urine metabolome fingerprint in dog for phenotypic classification	Viant, et., al	10.1007/s11306007009 20
2007	Metabonomic investigations of aging and caloric restriction in a life-long dog study	Wang, et., al	10.1021/pr060685n
2010	Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs	Beckmann et., al	10.1017/S000711450999300X
2012	NMR-based metabolomics study of canine bladder cancer	Zhang, et., al	10.1016/j.bbadis.2012.08.001
2015	Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea	Guard, et., al.	10.1371/journal.pone.0127259
2015	Consumption of Cooked Navy Bean Powders Modulate the Canine Fecal and Urine Metabolome	Forster, et., al.	10.2174/2213235X036661505 19234354
2017	The urine metabolome differs between lean and overweight Labrador Retriever dogs during a feed-challenge	Söder, et., al.	10.1371/journal.pone.0180086
2018	Species differences in bile acids I. Plasma and urine bile acid composition	Thakare, et., al	10.1002/jat.3644
2019	Untargeted metabolomic profiling of urine from healthy dogs and dogs with chronic hepatic disease	Lawrence, et., al.	10.1371/journal.pone.0217797
2020	Specific urinary metabolites in canine mammary gland tumors	Valko-Rokytovská, et., al.	10.4142/jvs.2020.21.e23
2020	Targeted Metabolomics With Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS) Highlights Metabolic Differences in Healthy and Atopic Staffordshire Bull Terriers Fed Two Different Diets, A Pilot Study	Moore, et., al	10.3389/fvets.2020.554296
2020	Urinary proteome and metabolome in dogs (Canis lupus familiaris): The effect of chronic kidney disease	Ferlizza, et., al	10.1016/j.jprot.2020.103795
2020	Varying Protein Levels Influence Metabolomics and the Gut Microbiome in Healthy Adult Dogs	Ephraim, et., al	10.3390/toxins12080517
2021	Combined Untargeted and Targeted Metabolomics Approaches Reveal Urinary Changes of Amino Acids and Energy Metabolism in Canine Babesiosis With Different Levels of Kidney Function	Kuleš, et., al	10.3389/fmicb.2021.715701

2021	Evaluation of Serum and Urine Amino Acids in Dogs with Chronic Kidney Disease and Healthy Dogs Fed a Renal Diet	Brunetto, et., al	10.3390/metabo11120844
2022	(1)H NMR based urinary metabolites profiling dataset of canine mammary tumors	Lee, et., al	10.1038/s41597-022-01229-1
2022	Profound Perturbation in the Metabolome of a Canine Obesity and Metabolic Disorder Model	Qu, et., al	10.3389/fendo.2022.849060
2022	Untargeted Metabolomics Identify a Panel of Urinary Biomarkers for the Diagnosis of Urothelial Carcinoma of the Bladder, as Compared to Urolithiasis with or without Urinary Tract Infection in Dogs	Tsamouri, et., al	10.3390/metabo12030200
2022	Urinary cortisol metabolites are reduced in MDR1 mutant dogs in a pilot targeted GC-MS urinary steroid hormone metabolome analysis	Gramer, et., al	10.1111/jvp.13050
2022	Comprehensive profiling of lipid metabolites in urine of canine patients with liver mass	Kida, et., al	10.1292/jvms.22-0191
2022	The profile of urinary lipid metabolites in healthy dogs	Kida, et., al	10.1292/jvms.22-0020
2023	Comparative plasma and urine metabolomics analysis of juvenile and adult canines	Wu, et., al	10.3389/fvets.2022.1037327
2023	Untargeted metabolomic profiles reveal widespread metabolic perturbations and identify candidate biomarkers in aminoaciduric canine hypoaminoacidemic hepatopathy syndrome	Loftus, et., al.	10.2460/ajvr.23.08.0186
2024	A longitudinal study of the blood and urine metabolome of Vipera berus envenomated dogs	Nicolaysen, et., al	10.1016/j.rvsc.2024.105287
2024	Candidate urinary biomarkers show promise for distinguishing between calcium oxalate versus struvite urolithiasis in dogs	Xu, et., al	10.2460/ajvr.23.09.0214

Table 3.3 Types and quality of reporting of urine collection methods in published dog urine metabolomics studies.

Year	Title	Authors	Consistent Collection Method?	Urine Collection Method(s)
2007	Validation of a urine metabolome fingerprint in dog for phenotypic classification	Viant, et., al	Yes	Free Catch
2007	Metabonomic investigations of aging and caloric restriction in a life-long dog study	Wang, et., al	No	Catheterization or cystocentesis
2010	Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs	Beckmann et., al	Yes	Free Catch
2012	NMR-based metabolomics study of canine bladder cancer	Zhang, et., al	Yes	Free Catch
2015	Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea	Guard, et., al.	Yes	Cystocentesis
2015	Consumption of Cooked Navy Bean Powders Modulate the Canine Fecal and Urine Metabolome	Forster, et., al.	No	Free Catch & Cystocentesis
2017	The urine metabolome differs between lean and overweight Labrador Retriever dogs during a feed-challenge	Söder, et., al.	Yes	Home Collection
2018	Species differences in bile acids I. Plasma and urine bile acid composition	Thakare, et., al	Unknown	Unknown
2019	Untargeted metabolomic profiling of urine from healthy dogs and dogs with chronic hepatic disease	Lawrence, et., al.	Yes	Cystocentesis
2020	Specific urinary metabolites in canine mammary gland tumors	Valko-Rokytovská, et., al.	No	Free Catch, Cystocentesis & Manual Compression
2020	Targeted Metabolomics With Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS) Highlights Metabolic Differences in Healthy and Atopic Staffordshire Bull Terriers Fed Two Different Diets, A Pilot Study	Moore, et., al	Unknown	Unknown
2020	Urinary proteome and metabolome in dogs (Canis lupus familiaris): The effect of chronic kidney disease	Ferlizza, et., al	Yes	Cystocentesis

2020	Varying Protein Levels Influence Metabolomics and the Gut Microbiome in Healthy Adult Dogs	Ephraim, et., al	Unknown	Unknown
2021	Combined Untargeted and Targeted Metabolomics Approaches Reveal Urinary Changes of Amino Acids and Energy Metabolism in Canine Babesiosis With Different Levels of Kidney Function	Kuleš, et., al	Yes	Free Catch
2021	Evaluation of Serum and Urine Amino Acids in Dogs with Chronic Kidney Disease and Healthy Dogs Fed a Renal Diet	Brunetto, et., al	No	Cystocentesis & Urethral Catheterization
2022	(1)H NMR based urinary metabolites profiling dataset of canine mammary tumors	Lee, et., al	No	Free Catch & Cystocentesis
2022	Profound Perturbation in the Metabolome of a Canine Obesity and Metabolic Disorder Model	Qu, et., al	Unknown	Unknown
2022	Untargeted Metabolomics Identify a Panel of Urinary Biomarkers for the Diagnosis of Urothelial Carcinoma of the Bladder, as Compared to Urolithiasis with or without Urinary Tract Infection in Dogs	Tsamouri, et., al	Unknown	Unknown
2022	Urinary cortisol metabolites are reduced in MDR1 mutant dogs in a pilot targeted GC-MS urinary steroid hormone metabolome analysis	Gramer, et., al	Yes	Home Collection
2022	Comprehensive profiling of lipid metabolites in urine of canine patients with liver mass	Kida, et., al	Unknown	Unknown
2022	The profile of urinary lipid metabolites in healthy dogs	Kida, et., al	Unknown	Unknown
2023	Comparative plasma and urine metabolomics analysis of juvenile and adult canines	Wu, et., al	Yes	Cystocentesis
2023	Untargeted metabolomic profiles reveal widespread metabolic perturbations and identify candidate biomarkers in aminoaciduric canine hypoaminoacidemic hepatopathy syndrome	Loftus, et., al.	No	Free Catch, Cystocentesis & Catheterization
2024	A longitudinal study of the blood and urine metabolome of Vipera berus envenomated dogs	Nicolaysen, et., al	Yes	Free Catch
2024	Candidate urinary biomarkers show promise for distinguishing between calcium oxalate versus struvite urolithiasis in dogs	Xu, et., al	No	Cystocentesis & Urethral Catheterization

3.3.4 Sample Storage

Storage conditions were documented in 21 of 25 studies (84%), with temperature distributions as follows: -80°C (16 of 25 studies, 64%), -28°C (2 of 25 studies, 8%), and 1 of 25 studies (4%) each for -20°C, -50°C, and -70°C. The remaining 4 of 25 studies (16%) did not report storage temperature information. Time between collection and storage was largely unreported, with 20 of 25 studies (80%) omitting this information. Among studies reporting this information (5 of 25 studies, 20%), timeframes ranged from approximately 30 minutes to 24 hours.

3.3.5 Sample Preparation - Thawing, Preparation & Temperature Control

Sample preparation varied widely, likely due to range of study designs and metabolomics platforms. Key methodological details were often omitted, with no studies comprehensively reporting thawing techniques or durations. While 3 of 25 studies (12%) specified sample thawing temperature, they omitted timeframe. Incomplete reporting of reagents, reagent/chemical quantities, equipment parameters, and/or container specifications occurred in 25 of 25 studies (100%).

3.3.6 Temperature Control - Before and During Data Acquisition

Most studies (21 of 25 studies, 84%) failed to specify whether samples were analyzed immediately after preparation or after a delay, and did not report interim storage conditions. The remaining 4 of 25 studies (16%) indicated samples were stored between preparation and data acquisition but omitted duration. Additionally, 21 of 25 studies (84%) did not report whether samples were temperature-controlled during data acquisition, while 4 of 25 studies (16%) reported storage temperatures ranging from 4°C to 15°C while awaiting acquisition.

3.4 Discussion

We found substantial methodological gaps and inconsistencies that compromise research reliability and reproducibility in canine metabolomics research. The findings in this review are particularly timely given increasing traction of metabolomics approaches in veterinary medicine supported by the proportion of studies (16 of 25, 64%) being published between 2020-2024.

One of the most critical findings of this review was lack of collection reporting across studies with only 3 of 25 studies (12%) providing complete documentation of collection methods and container specifications. This inconsistency introduces potential for variability that may substantially impact metabolomic profiles of urine samples. The use of different collection methods, free-catch, cystocentesis, and catheterization, presents potential challenges due to varying levels of contamination from microflora of distal urogenital tract, and possible prostatic, vaginal, and cellular secretions or debris depending on the method (Coffey et al., 2023; Das et al., 2017). Studies employing mixed collection methods or failing to report methods entirely raise additional concerns about data reliability and cross-study comparability.

Sample handling procedures demonstrate equally problematic variation, with 21 of 25 studies (84%) reporting insufficient information to replicate or validate findings. More than half of the studies (15 of 25 studies, 60%) did not report any sample handling procedures, while one study (4%) provided insufficient detail for replication. This gap in methodological transparency is substantial since handling steps such as centrifugation and filtration directly impact urine metabolome stability (Morello et al., 2015; Patrizia et al., 2011; Saude & Sykes, 2007). Variability of reported procedures ranging from centrifugation to antimicrobial addition, introduces methodological variables that complicate discernment between true biological variation and procedure-induced artifacts.

Storage condition documentation, while more consistent for temperature reporting (21 of 25 studies, 84%) revealed gaps regarding the timeframe between collection and storage. Absence of this information in 20 of 25 studies (80%) highlights another limitation since delays may lead to metabolite degradation, particularly those sensitive to temperature changes (Giskeødegård et al., 2019). Studies failing to report this information leaves uncertainty regarding sample integrity and metabolomics data reliability.

The most common documentation gap involved sample preparation. Complete absence of thawing protocols and incomplete reporting of preparation details across all studies fundamentally undermines reproducibility efforts. While methodological variation between studies may be justified based on analytical platforms and research objectives, lack of transparency in reporting prevents validation of findings and limits advancement in the field.

These findings highlight stark contrast with established standards in human urine metabolomics where numerous research studies have produced standardized protocols and recommendations that enhance reproducibility (Wishart, 2019). The variability and documentation gaps identified herein demonstrate urgent need for standardized protocols in veterinary metabolomics research. These protocols should incorporate best practices from human metabolomics while addressing veterinary-specific and species-specific challenges.

Widespread inconsistency in methodological reporting emphasizes need for enhanced transparency in research documentation. Scientific journals and peer reviewers must emphasize need for comprehensive and transparent reporting of all methodological steps, as none of the studies in this review reported enough details for true repeatability. While space constraints may limit method sections, supplementary materials provide opportunity for detailed protocol

documentation to ensure reproducibility and generation of reliable data for clinical application and future investigations.

Establishing standardized protocols and enhancing reporting transparency is required for generating high-quality, reproducible data that contributes to understanding canine health and disease while ensuring findings are valuable for translational research. This review demonstrates need for veterinary metabolomics to adopt systematic procedures that align with established human metabolomics standards while accounting for species specific considerations.

3.5 Future Directions

Future advancement of dog urine metabolomics requires collaborative and coordinated efforts across the human and veterinary research communities. Development of standardized protocols must address technical aspects, including optimal collection methods, handling procedures, and storage conditions, and practical aspects unique to veterinary medicine. Systematic investigation of metabolite stability under various collection and storage conditions is needed to establish evidence-based guidelines. Multi-center validation studies comparing different collection methods would help to determine impact of methodology on metabolomic profiles while also accounting for breed, age, and disease-specific variations. Additionally, establishment of centralized metabolomics databases specifically for veterinary species, including dogs, would enhance cross study comparability and biomarker validation. These efforts, combined with improved transparency in methodology reporting, will advance reliability of dog urine metabolomics and strengthen impact for both veterinary medicine, nutrition and comparative research initiatives.

CHAPTER 4

INVESTIGATION OF STABILITY OF Nε-CARBOXYMETHYLLYSINE (CML) and Nε-CARBOXYETHYLLYSINE (CEL) IN FREE-CATCH DOG URINE[1]

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To be submitted to a peer-reviewed journal

Contributors

Nicole R. Cammack	- principal investigator, project design, sample collection, preparation, statistical analyses, and writing
Joseph W. Bartges	- project design, statistical analyses, and editing
Stephanie Archer-Hartmann	- data analyses and editing
Parastoo Azaidi	- data analyses and editing

Abstract

Advanced glycation end products (AGEs) such as Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL) are implicated in chronic disease processes in humans and may serve as biomarkers of dietary exposure and metabolic health. Urinary measurement of AGEs is of interest due to its non-invasive nature and relevance to biobanking and field-based sample collection. However, stability of AGEs in urine under common handling conditions is poorly defined. This study evaluated short-term stability of CML and CEL in canine urine stored at room temperature (20 °C) for up to 168 hours prior to freezing at -80 °C. Midstream free-catch urine samples from eight healthy dogs were aliquoted, stored at defined intervals, and analyzed in duplicate using LC-MS with isotope-labeled standards. Results demonstrated minimal detectable changes in CML or CEL concentrations, or in the CML/CEL ratio, over the 7-day ambient storage period. Inter-replicate agreement was high, and regression and non-parametric analyses showed no association between storage duration and analyte concentration. These findings suggest CML and CEL exhibit minimal change in canine urine for up to 168 hours at room temperature, supporting the feasibility of delayed processing in field settings and

retrospective studies. Evaluation of additional AGE species and varying storage conditions will further inform best practices for sample handling in veterinary and comparative biomedical research.

4.1 Introduction

There is growing interest in the role of advanced glycation end products (AGEs) in health and disease in humans and dogs (Ottka et al., 2021). AGEs, result from non-enzymatic reactions between reducing sugars and proteins or nucleic acids (Poulsen et al., 2013). Reactions occur endogenously during normal metabolism and exogenously during thermal processing of dietary ingredients (Twarda-Clapa et al., 2022). Over 20 AGEs have been found within human biofluids, tissues and in food products, and commonly categorized into groups based on chemical structure and fluorescence properties (Perrone et al., 2020; Twarda-Clapa et al., 2022). Dietary AGEs are the main source of the AGE body pool in addition to endogenous AGE production, and elimination of AGEs is via gastrointestinal and urinary excretion (Palaseweenun et al., 2021). Approximately one-third of gastrointestinal-absorbed AGEs are excreted in urine within 48 hours in healthy humans (Koschinsky et al., 1997), although excretion may be 5% lower with renal disease.

In humans, consumption of ultra-processed diets with high amounts of AGEs are associated with chronic diseases such as diabetes mellitus, arthritis, cancer, chronic kidney disease, and cognitive dysfunction (Hoffman et al., 2018). Dogs share similar chronic diseases and environmental exposures, notably consumption of ultra-processed diets. Commercial pet foods contain similar levels of AGEs found in ultra processed human foods, although dogs consuming commercial diets consume 122-fold higher AGEs than humans consuming a Western diet on a metabolic body weight basis (van Rooijen et al., 2014). This is attributed to dogs being fed the same diet (e.g. kibble, canned) often for a lifetime with little to no variety. Additional studies demonstrate that dogs consuming high AGE containing diets may serve as translational models (Bridglalsingh et al., 2024; Oba et al., 2022).

AGEs may be measured in diet, serum, and urine (Scheijen et al., 2018). Biobanking initiatives of samples such as urine are becoming more frequent (Alexander et al., 2023; Labadie et al., 2022; LaLonde-Paul et al., 2023; Sándor et al., 2022). Different metabolite classes exhibit varying sensitivity to storage conditions such as time, temperature fluctuations, light, and oxidation (Aurelie et al., 2015; Braisted et al., 2024; Lehmann, 2021). As such, time delays, temperature fluctuation, and contamination prior to storage and data acquisition may result in metabolite instability in biosamples (Rotter et al., 2016).

While AGEs in food products are generally considered stable over time, their stability in urine are poorly characterized. Underestimation or overestimation of true AGE concentrations due to lack of standardization for collection and storage effecting sample stability result in inaccurate research findings (Rotter et al., 2016). Detection and quantification of N-ε-carboxymethyllysine (CML) and N-ε-carboxyethyllysine (CEL), which are nonfluorescent, non-cross-linked AGEs occurs commonly (Twarda-Clapa et al., 2022). CML arises from multiple pathways, including oxidation of Amadori products, glyoxal-mediated reactions from carbohydrate or lipid oxidation, and free radical processes (Han et al., 2013). CEL is primarily formed through the reaction of lysine residues with methylglyoxal or triose-phosphate, both reactive dicarbonyl compounds generated during lipid peroxidation and sugar degradation (Srey et al., 2010). Formation is influenced by factors such as temperature, time, moisture content, and the presence of reactants like sugars, lipids, and proteins, as well as potential inhibitory compounds (Poulsen et al., 2013). CML and CEL are considered important biomarkers for assessing AGE exposure and the endogenous AGE pool, and have been associated with numerous conditions in humans (Lane et al., 2024; Martinez-Moral & Kannan, 2022). Although roles in canine health are not defined, these AGEs are believed to contribute to chronic diseases

in dogs similar to humans, and their presence and relationship to diet have been investigated in canine urine (Bridglalsingh et al., 2024; Palaseweenun et al., 2021). The lack of stability data on AGEs limits confidence in cross-study comparisons and potentially undermines data interpretation in large-scale or retrospective research. Stability of CML and CEL has been evaluated in plasma and serum where collection tube type, delayed processing, storage temperature, and multiple freeze–thaw cycles) do not significantly alter measured concentrations (Hull et al., 2013). CML stability has been investigated in food systems, demonstrating storage conditions significantly influence AGE accumulation. Specifically, CML levels in skim milk powder increased substantially during prolonged storage under higher humidity and temperature, highlighting AGEs can continue to form post-processing if conditions are unfavorable (Aalaei et al., 2017). The disparity of AGE stability between foods and biofluid demonstrate need for validation studies assessing stability of CML and CEL in urine. Furthermore, factors such as cellular debris and bacterial contamination may contribute to sample instability in urine (Budde et al., 2016; Coffey et al., 2023; Ghini et al., 2019; Patrizia et al., 2011; Saude & Sykes, 2007), demonstrating need for standardized processing protocols in AGE research.

The purpose of this study was to evaluate short-term stability of CML and CEL in canine urine stored at -80 °C after standing at 20 °C (room temperature) for various lengths of time. Given potential for concentration variability during routine sample handling, we aimed to determine if CML, CEL, and CML/CEL ratio remain stable over a 168-hour (7-day) at 20 °C before placing in -80 °C. The timeframe reflects realistic and extended delays often occurring between sample collection and analysis, particularly in field studies or shipping samples to centralized laboratories or biobanks. We hypothesized that concentrations of CML, CEL, and CML/CEL ratio would show minimal change over time when samples were held at 20 °C prior

to -80 °C storage. However, statistical analyses were conducted to test whether any significant changes occurred across time points.

4.2 Methods

Sample Collection, Processing & Storage

Midstream free-catch urine samples (90-120 mL) were collected from eight clinically healthy dogs[a] using sterile 120 mL polypropylene specimen containers (Dynarex, item #4353) by trained personnel. Post collection, samples were placed on ice and transported within 10 minutes to the laboratory. Upon arrival, samples were vortexed for 30 seconds (VWR Vortex Mixer VWR International, Radnor, PA), and 1 mL aliquots were transferred into Eppendorf Flex-Tube microcentrifuge polypropylene tubes (Eppendorf, Hamburg, Germany, item #022363531) and kept on ice. Each sample was divided into eleven duplicate aliquots for storage (0, 1, 3, 6, 12, 24, 36, 48, 72, 120, and 168 hours). Aliquots were centrifuged (2000 RCF, 5 minutes, 4 °C, via Eppendorf 5417 C), and supernatant transferred into sterile 2 mL cryovials for storage. Hour 0 aliquots were immediately frozen at −80 °C and remaining samples kept at 20 °C with transfer to −80 °C at designated time points. Quantification of CML and CEL was performed using liquid chromatography–mass spectrometry (LC-MS) as previously described (Bridglalsingh et al., 2024). An overview of sample collection, processing, and storage is summarized in figure 4.1

4.2.2 Reagents and Standards

Formic acid (≥99% purity), acetonitrile (ACN), and distilled water, all LC-MS grade (Thermo Fisher Scientific). Internal standards were Nε-(1-carboxymethyl)-L-lysine-d_3 (CML-d_3, Cayman Chemical, Item #: 26785) and Nε-(1-carboxyethyl)-L-lysine-d_4 (CEL-d_4, Iris Biotech, Product

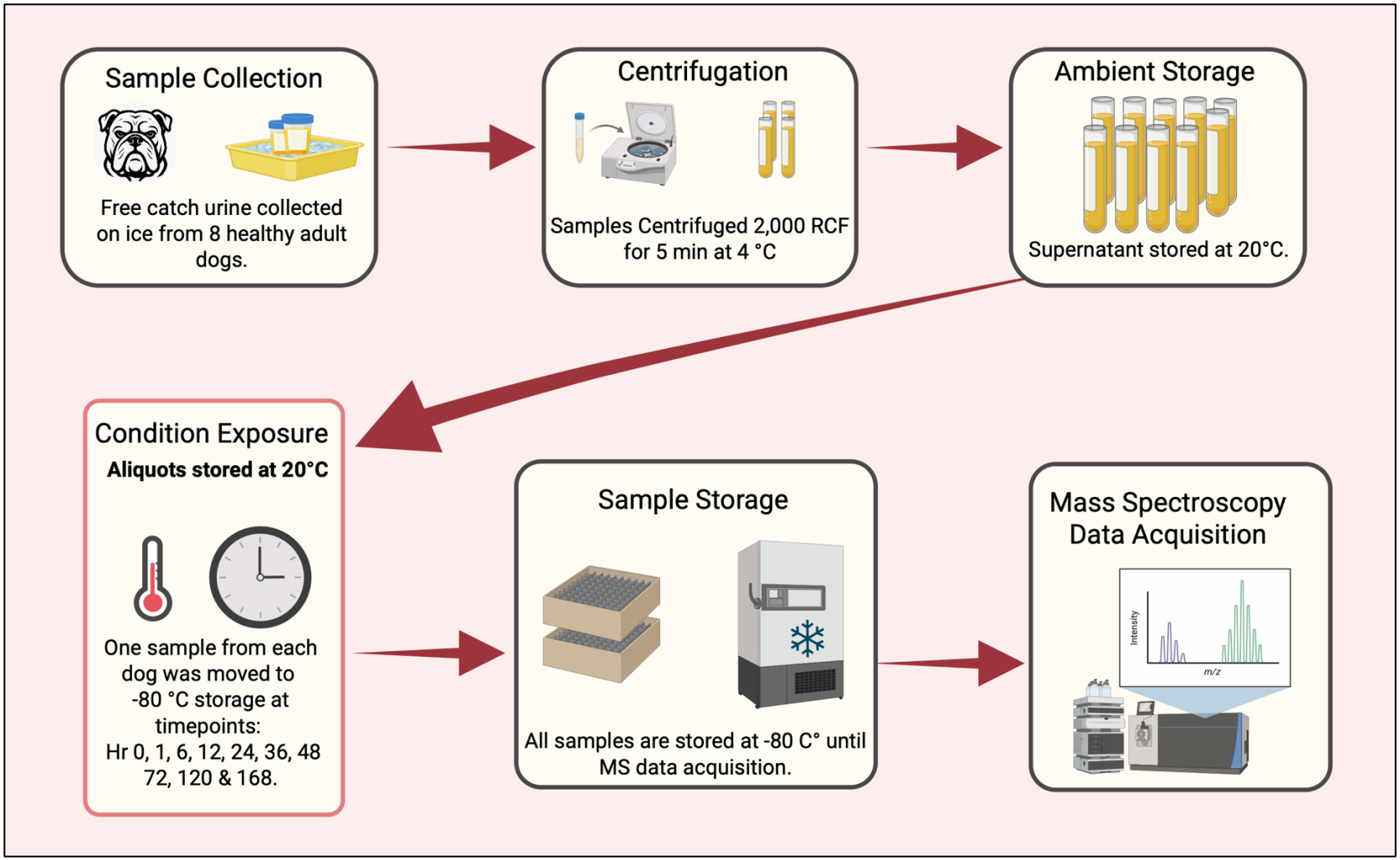


Figure 4.1. Overview of Dog Urine Collection, Handling & Storage. Created in https://BioRender.com

Code: HAA2941), each prepared at 10 µM in 0.1% formic acid. All other reagents were of analytical or LC-MS grade.

4.2.3 Mass Spectrometry Analysis

Quantification of CML and CEL was performed as described by Bridglalsingh et al. (2024) with minor modifications[b]. Aliquot of 0.5 mL urine was filtered with 10-kDa molecular weight cutoff centrifugal filter (Pall Corporation). Filtrate diluted 50× into solution containing 10 µM CML-d_3 and 10 µM CEL-d_4 in 0.1% formic acid. Analysis performed using Orbitrap QExactive Mass Spectrometer (Thermo Fisher Scientific) coupled to Vanquish UPLC system (Thermo Fisher Scientific) equipped with electrospray ionization (ESI) operating in positive ion mode. Separation was achieved on a Millipore SeQuant® ZIC-HILIC column (3.5 µm, 150 × 2.1 mm) using stepwise solvent gradient. Solvent A was 100% LC-MS grade water with 0.1% formic acid, and solvent B was 100% LC-MS grade acetonitrile with 0.1% formic acid. The flow rate was 350 µL/min, with elution profile as follows: 0–2 min: isocratic at 90% B, 2–3 min: linear gradient from 90% to 65% B, 3–5 min: isocratic at 65% B, 5–7 min: linear gradient from 65% to 40% B, 7-10 min: isocratic at 40% B, 10-12 min: linear gradient from 40% to 90% B, 12–28 min: isocratic at 90% B. Samples were injected at volume of 1 µL. Ion detection was performed using selected ion monitoring (SIM) with m/z values of 205/208 for CML/CML-d_3 and 219/223 for CEL/CEL-d_4. Data acquired with Thermo Fisher's Excalibur software. Quantification was based on ratio of extracted ion peak areas of internal standard (CML-d_3 or CEL-d_4) to corresponding biological AGE peak.

For analytical reproducibility assessment, samples were analyzed twice (N1 and N2) using identical LC-MS/MS conditions including the Vanquish UPLC system, and kept in thermostated autosampler at 10 °C. N1 and N2 were processed independently on separate days

using same instrumentation, columns, mobile phases, and protocol. Both replicates were used in downstream analysis to evaluate inter-assay agreement and temporal stability of CML and CEL concentrations in canine urine. Raw data from both runs can be found in Appendix B.

4.3 Analysis

To assess inter-replicate variability for CML and CEL, concentrations from duplicate runs (n = 176; 88 per sets of replicates) were compared using descriptive statistics and the Kruskal–Wallis test, given non-normal distribution of values based on the results of the Shapiro-Wilk test of normality. Boxplots were used to visualize inter-replicate distributions across all dogs. Aggregated CML and CEL concentrations (n = 176 each) were evaluated via histograms with overlaid kernel density estimates to assess distribution characteristics as well as guide statistical tests. Finally, CML/CEL ratios were calculated and similarly visualized.

To evaluate the influence of RT storage duration on AGE concentrations, data from both replicates were included in linear regression models for CML, CEL, and the CML/CEL ratio, with time (hours) as the independent variable. The magnitude of observed changes over time was evaluated by examining regression slopes and their confidence intervals. Model fit was assessed using R^2 and ANOVA. Residuals were evaluated for normality and homoscedasticity using Shapiro-Wilk tests and residual plots. CML, CEL, and CML/CEL ratios were further analyzed using non-parametric methods to examine the magnitude of changes across time points. Medians per group were visualized via color-coded boxplots by dog. Kruskal-Wallis was used to assess central tendency. Lastly, Steel-Dwass-Critchlow-Fligner post hoc comparisons were employed to control for multiple testings.

Normalization to creatinine or specific gravity was not applied in our study, as the objective was to assess absolute analyte stability under controlled conditions, independent of physiological dilution factors.

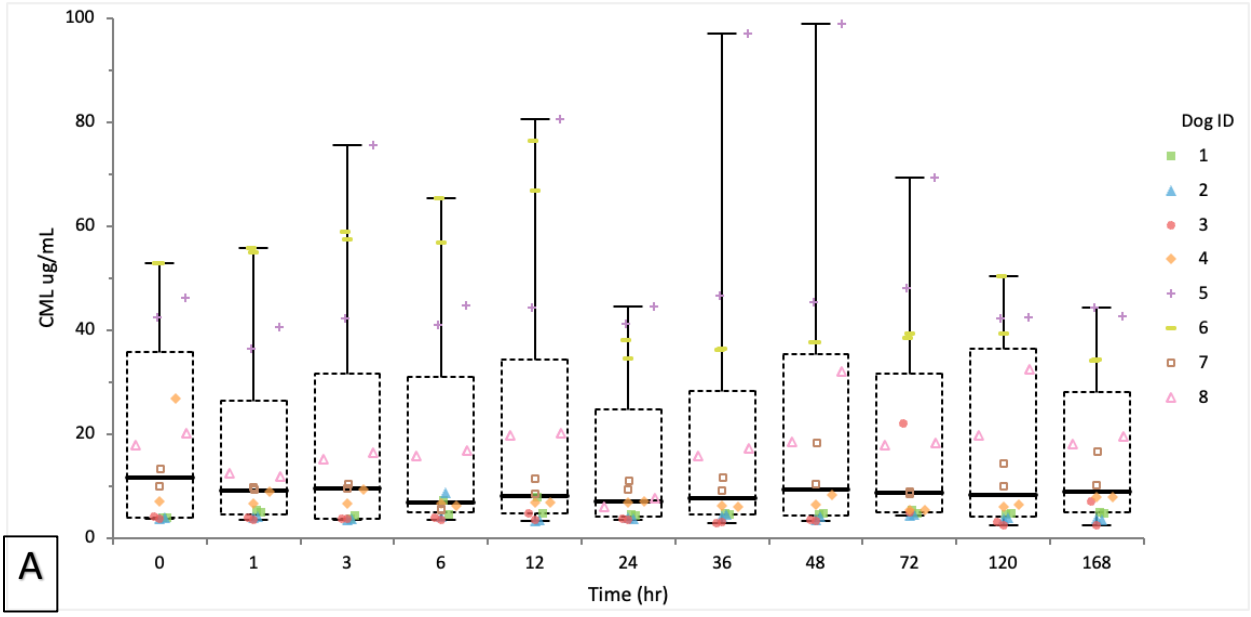
4.4 Results

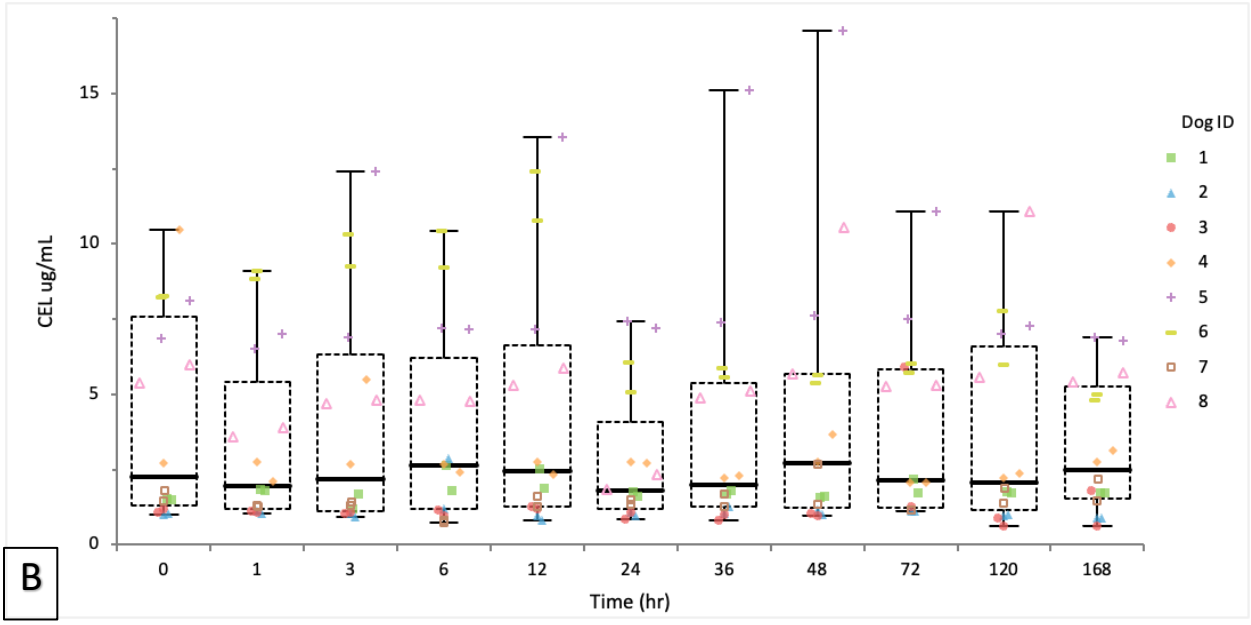
Comparison between N1 and N2 indicated high reproducibility for CML and CEL quantifications. Medians concentrations of 9.13 µg/mL (IQR: 4.65-33.46) and 7.00 µg/mL (IQR: 4.33-28.29) for CML, and 2.36 µg/mL (IQR: 1.28–5.95) and 1.86 µg/mL (IQR: 1.20-5.49) for CEL were observed. Concentrations were highest in the same dogs across both replicates for each AGE (CML: 98.81 vs. 66.74 µg/mL; CEL: 17.07 vs. 10.76 µg/mL). Kruskal-Wallis testing found no statistically significant differences between replicates (CML: $H = 1.52$, $p = 0.218$; CEL: $H = 1.29$, $p = 0.256$), indicating consistent analytical performance. Boxplots displayed overlapping distributions with consistent interquartile ranges (Figure 4.2A and 4.2B). CML aggregate values ($n = 176$) were positive and concentrated between 2-20 µg/mL with peak near 10 µg/mL and outliers >50 µg/mL in the upper range of the plot. Additionally, CEL values were also positively skewed, with majority <2 µg/mL and a maximum near 16 µg/mL. Histograms for each AGE deviated from normality, suggesting inter-individual variability and temporal heterogeneity.

Linear regression analysis revealed minimal changes in urinary concentrations over time for both AGEs. The slope was -0.0138 µg/mL/hr (95% CI: -0.0727 to 0.0451, $p = 0.6447$), intercept 23.51 µg/mL (95% CI: 19.46 to 27.57), and adjusted $R^2 = 0.001$ for CML. For CEL, the slope was −0.00236 µg/mL/hr (95% CI: -0.0114 to 0.0072, $p = 0.6269$), intercept 4.60 µg/mL (95% CI: 3.94 to 5.26), and adjusted $R^2 = -0.004$. Additionally, F-tests (CML: $F = 0.21$; CEL: $F = 0.24$) indicated that time did not explain meaningful variance in AGE concentrations. Residual

plots did not show heteroscedasticity or autocorrelation. Lastly, Shapiro-Wilk testing confirmed non-normality (CML: $W = 0.76$, $p < 0.0001$; CEL: $W = 0.87$, $p < 0.0001$).

For the CML/CEL ratio, linear regression analysis showed minimal change over the storage period. The slope was 0.001638 (95% CI: -0.003449 to 0.006725; $p = 0.5259$), intercept of 3.60 µg/mL (95% CI: 3.37 to 3.82) and adjusted $R^2 = 0.002$. The F-test ($F = 0.40$) indicated that time did not explain meaningful variance in the CML/CEL ratio. Residual plots showed no heteroscedasticity or autocorrelation, with Shapiro-Wilk also confirming non-normality ($W = 0.90$, $p < 0.0001$) for the CML/CEL ratio (Figure 4.2C).





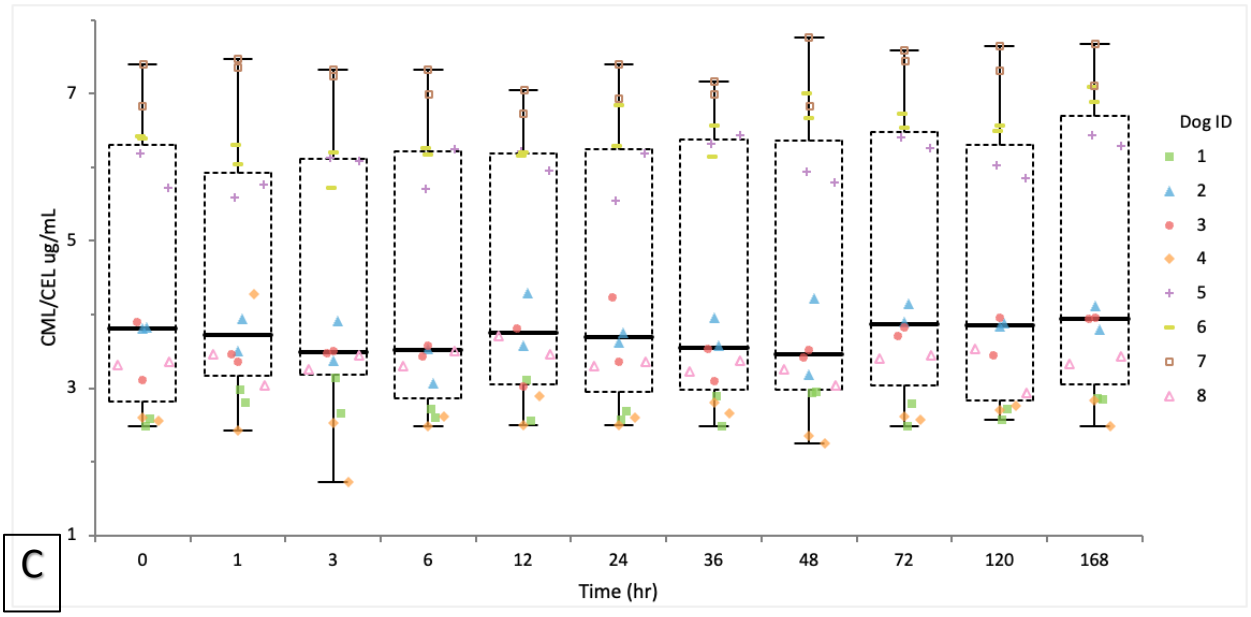


Figure 4.2 Temporal changes in CML, CEL, and the CML/CEL ratio in canine urine stored at room temperature over 168 hours: Boxplots display concentrations of (A) Nε-carboxymethyllysine (CML), (B) Nε-carboxyethyllysine (CEL), and (C) the CML/CEL ratio across eleven time points (0, 1, 3, 6, 12, 24, 36, 48, 72, 120, and 168 hours). Data represent combined technical replicates (n = 176 per analyte). Individual dogs are color-coded to illustrate inter-individual variability. Horizontal black lines indicate group medians. Across all analytes,

median values showed minimal variation over time, with Kruskal–Wallis tests indicating no statistically significant differences across time points (CML: $p = 0.9991$; CEL: $p = 0.9993$; CML/CEL ratio: $p = 0.9993$). These results show that CML and CEL concentrations exhibited minimal observable change within urine of individual dogs under ambient storage conditions for up to seven days.

4.5 Discussion

Our study demonstrated that urinary concentrations of CML and CEL showed minimal detectable change within urine of individual dogs under ambient storage conditions for up to seven days, prior to storage at −80 °C. The observed changes in concentrations over time were minimal and within the range of analytical variability, suggesting these analytes may remain relatively stable during common sample handling delays. This data establishes baseline data to guide initial sample handling protocols in canine urine AGE research, enabling more consistent and reliable biomarker analyses across field studies and biobanking efforts.

Determining short-term stability of AGEs in urine is important for expanding biobanking capabilities and to ensure sample reliability even when subjected to storage delays such as field-collected or remotely shipped samples. In large-scale studies and multisite collaborations, immediate access to −80 °C storage is often unrealistic. The minimal observed changes over 168 hours suggest that urinary AGE measurements may remain reliable even when samples are subject to logistical delays, such as mailing, temporary refrigeration, or ambient storage prior to processing. These findings improve feasibility of longitudinal studies, data confidence and retrospective biomarker analyses where urine AGE profiles are of interest.

To the authors' knowledge, no studies have investigated AGE stability in urine. Existing research primarily focuses on plasma or serum, where delayed processing and freeze–thaw cycles appear to exert minimal influence on CML and CEL concentrations. However, plasma and urine differ significantly in matrix composition and susceptibility to degradation pathways (Schlosser et al., 2023). Our findings provide urine-specific validation that supports application of urinary AGE research and related dietary and disease investigations in comparative biomedical research.

The current study's strengths include modeling realistic handling conditions, use of MS with isotope-labeled standards, and inclusion of multiple time points extending to 168 hours. However, limitations include evaluation of only two AGE species, a single controlled temperature condition, and sampling restricted to healthy adult dogs. Results may not directly extrapolate to diseased populations or samples exposed to greater environmental variation. Additionally, while our statistical analyses detected no significant changes over time, the study design was not specifically powered to demonstrate equivalence or stability within predefined acceptable limits. We did note that the variance between dogs was greater for CML and CEL, and indicates need for future work to determine reference ranges. Future work in this area should also extend stability assessments to include additional AGEs, including argpyridamine, methylglyoxal-derived hydroimidazolone (MG-H1), pyrraline, pentosidine, and others to build a more comprehensive profile of AGE behavior in urine.

A major challenge in metabolomics and similar biomarker investigations in urine such as this study stem from the influence of cellular components and bacterial contamination originating from the distal urogenital tract (Coffey et al., 2023; Das et al., 2017). The presence of cellular debris is recognized to alter the metabolomic profile by introducing enzymatic activity and shifting pH (Budde et al., 2016), while bacterial contamination has been shown to modify urinary metabolic profiles through both metabolic activity and degradation processes (Ghini et al., 2019; Maher et al., 2007; Morello et al., 2015; Saude & Sykes, 2007). Mishandling during collection or processing may exacerbate these risks, potentially compromising sample integrity and stability (Ghini et al., 2019; Patrizia et al., 2011). Our sample processing technique utilized gentle centrifugation to remove cellular debris without inducing cellular breakage. This practice reduces risks of pH alteration, enzymatic degradation, or oxidative processes that can impact

urine metabolite stability. Although filtration was not performed and low-level bacterial contamination cannot be excluded, the chemical robustness of CML and CEL suggests these factors likely exerted minimal influence in the present study. Future studies targeting broader or more labile metabolite classes may benefit from incorporating these steps to further enhance pre-analytical integrity. As such, broader classes of metabolites relevant to oxidative stress, inflammation, and metabolic health also warrant evaluation in this context. Additional temperature conditions, (e.g. uncontrolled fluctuations in temperature during mailing, storage on ice, and refrigeration) should be systematically evaluated to guide more robust pre-analytical recommendations for veterinary and human biobanking initiatives.

Footnotes

a - Animal Care and Use Approval:
This protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia in compliance with the Animal Welfare Act.

b - AGE analysis was performed at the Complex Carbohydrate Research Center and was supported in part by the National Institutes of Health (NIH)-funded R24 grant (R24GM137782) to Parastoo Azadi

CHAPTER 5

CONSIDERATIONS FOR METABOLOMICS STUDIES IN CANINE MODELS: A PILOT ANALYSIS OF DOG URINE STABILITY[1]

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Contributors

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Abstract

This pilot study evaluated the short-term stability of voided canine urine samples under two common storage conditions, refrigeration (4 °C) and room temperature (20 °C), using untargeted one-dimensional proton nuclear magnetic resonance (1D ^{1}H NMR) spectroscopy. A total of 320 samples were prepared from sixteen clinically healthy dogs and analyzed over 72-hours. Following spectral quality assessments and alignment diagnostics, data from six dogs were retained for final analysis. Among 438 detected features, 107 consistently aligned peaks were

analyzed for intensity variation over time. Samples stored at 4 °C exhibited greater metabolite stability than those stored at 20 °C, particularly beyond 24 hours. Instability, defined as a >30% change from baseline, was more frequent in room temperature conditions. Mixed-effects models identified a small subset of features exhibiting significant time or temperature effects reinforcing the role of storage conditions in preserving sample integrity. Notably, extensive inter-individual metabolic variability among dogs exceeded effects attributable to storage. Despite sample size and resolution constraints, this study offers insight into short-term metabolite stability and reinforces the need for species-specific protocols to improve reproducibility and advance veterinary metabolomics.

5.1 Introduction

Metabolomics has transformed disease research by enabling detailed profiling of metabolic perturbations. In human medicine, it is increasingly integrated into diagnostics, supported by robust protocols for sample collection, processing, and storage. These frameworks, resulting from numerous stability investigations, have demonstrated pre-analytical handling directly impacts data integrity (Abdul-Hamid et al., 2015; Aurelie et al., 2015; Braisted et al., 2024; Delanghe & Speeckaert, 2016; Gika et al., 2008; Gika et al., 2007; Patrizia et al., 2011; Rainer Lehmann, 2020; Raúl et al., 2020; Rist et al., 2013; Wishart, 2019). In contrast, veterinary metabolomics has not established comparable standardizations. This represents a missed opportunity, as dogs share over 360 diseases and conditions with humans, including diabetes mellitus, obesity, cancer, arthritis, and cognitive decline, making them a valuable translational model (Carlos et al., 2020; Parker & Ostrander, 2005; Patterson, 2000; Shearin & Ostrander, 2010; Wayne & Ostrander, 2007). However, canine metabolomics studies frequently exhibit significant methodological inconsistencies, particularly regarding urine sample handling limiting the reliability, reproducibility, and interpretation of metabolite data as detailed in a previous chapter in this dissertation.

Sample stability is especially susceptible to degradation due to bacterial contamination from commensal flora and residual cellular material from the distal urogenital tract (Coffey et al., 2023; Das et al., 2017). Cellular components and bacteria have been shown to alter metabolic profiles in humans (Budde et al., 2016; Ghini et al., 2019; Maher et al., 2007; Morello et al., 2015; Saude & Sykes, 2007). Inconsistent handling practices such as delays in cooling or inappropriate storage temperatures further compound these effects. Although veterinary research often assumes comparability with human protocols, absence of validated, species-specific

methodologies introduces variability that can introduce challenges with data collection and analysis subsequently confounding biomarker discovery.

This study aimed to evaluate stability of voided canine urine samples across time and temperature conditions commonly encountered in veterinary research and clinical practice. The initial design employed untargeted NMR-based metabolomics to compare storage at 4 °C and 20 °C over 72-hours. We restructured the study to focus on 1D NMR-derived stability patterns and extract practical methodological insights from the workflow. These adjustments, summarized in Table 5.1, reveal key decision points and offer transparent guidance for future canine urine metabolomics research.

Without protocol development, the field remains limited in its ability to compare findings across studies or contribute meaningfully to translational research. While this chapter does not attempt to formalize best practices, findings presented provide a preliminary point for understanding pre-analytical variability in canine urine metabolomics. By contextualizing these findings within wider efforts to standardize veterinary metabolomics workflows, this study contributes foundational knowledge to support future protocol refinement and improve reproducibility in comparative research.

5.2 Methods

Study Population & Sample Collection

Sixteen clinically healthy dogs[a] (age >2 years, body condition score 4/9-7/9) were enrolled. Dogs were excluded with any history of urinary disease, antibiotic treatment or anesthetic procedures within 4 weeks prior to collection, or presence of serious systemic disease (e.g. cancer, diabetes mellitus, chronic kidney disease).

Midstream free-catch urine samples (90-120mL) were collected in sterile 120mL containers (Dynarex polypropylene specimen container, item # 4353), either immediately prior to entry into the Veterinary Medical Center at the University of Georgia by owners or research personnel. Each sample was immediately vortexed for 30 seconds and placed on ice while divided into twenty 4 mL aliquots (Corning® polypropylene 5 mL external thread cryogenic vials, item #66021-980) according to existing laboratory protocol (https://www.protocols.io/view/sample-prep-urine-nan-b8ubrwsn/materials).

5.2.2 Storage Protocol

At baseline (hour 0), one 4 mL aliquot from each dog was transferred immediately to -80°C storage. Remaining aliquots were equally divided between refrigeration (4°C) and room temperature (20°C) storage conditions. Paired aliquots from each storage temperature were subsequently transferred to -80 °C at 1, 3, 6, 9, 12, 24, 36, 48, and 72 hours post-collection, yielding 320 total samples (20 aliquots per dog: 2 temperatures x 10 timepoints).

5.2.3 Sample Preparation

Prior to sample preparation and subsequent analysis, all samples were thawed on ice for 2 hours, vortexed at 4 °C for 30 seconds and placed back on ice. Preparation conducted on 320 samples from 16 dogs, with randomization and organization in 96-well plates of 5mm NMR racks (Bruker BioSpin, Billerica, MA, part # Z112273). Each rack incorporated quality control measures consisting of buffer blanks (n=6), rack-specific internal pools (n=3), and human urine external pools (n=3), totaling 368 samples. Samples were thawed on ice for 2 hours then vortexed at 4 °C for 30 seconds using a VWR Vortex Mixer (VWR International, Radnor, PA). One mL aliquots were transferred to 1.5 mL Eppendorf Flex-tube microcentrifuge polypropylene

tubes (Eppendorf, Hamburg, Germany, item # 022363531) and centrifuged for 5 minutes at 12,000 g at 4C using an Eppendorf 5417 C centrifuge.

Following centrifugation, 540 µL of supernatant was transferred to new 1.5mL Eppendorf tubes and combined with 60 µL of potassium phosphate buffer. Buffer preparation consisted of dissolving 20.4 g KH_2PO_4 (Sigma-Aldrich, St. Louis, MO) in 80 mL deuterium oxide (D_2O, Cambridge Isotope Laboratories, Tewksbury, MA), followed by the addition of 24.9 mg DSS-D6 and 13 mg NaN_3 in 10 mL D_20. Solutions were combined and mixed using a magnetic stirrer (Corning Life Sciences, Corning, NY) for 5 minutes at setting 4, with pH adjusted to 7.4 using KOH pellets (Sigma-Aldrich). Final volume was adjusted to 100 mL with D_2O, and buffer was stored at 4°C until use.

Sample-buffer mixtures were vortexed for 2 minutes at 4°C and centrifuged at 12,000 g for 2 minutes. Following centrifugation, 590 µL of each sample was transferred to 5 mm Bruker NMR tubes (Bruker BioSpin), capped with polyoxymethylene (POM) balls (Bruker BioSpin), and placed in a SampleJet autosampler (Bruker BioSpin) maintained at 4°C. Temperature was monitored throughout. Sample preparation was completed within 2 hours of thawing, and NMR data acquisition began within 20 minutes of sample preparation completion. Chain of custody was maintained throughout and sample positions in 96-well plates were documented for traceability.

This design enabled systematic evaluation of both temperature and temporal effects on urine sample stability under conditions commonly encountered in clinical research settings.

5.2.4 Data Acquisition & Processing

NMR data were acquired using an Advance III HD 600 MHz Bruker NMR spectrometer equipped with a 5-mm TCI cryoprobe (Bruker). One-dimensional (1D) ^1H NMR experiment

noesypr1d (Bruker) was performed on all samples. After data collection, samples were stored in the same 5 mm NMR tubes at -20 °C for future 2D data acquisition.

Raw NMR data underwent initial phase and baseline correction using batch processing scripts via MestraNova (MNOVA) v. 14.2 processing scripts. Data were subsequently reprocessed using NMRPipe though the NMRbox cloud-based platform. Data were also processed using Spectral Automated NMR Decomposition (SAND) software that decomposes NMR data and quantifies overlapping peaks. SAND is an automated processing that increases efficiency by avoiding manual peak picking and binning NMR data, resulting in a refined dataset that streamlines statistical analysis and avoids traditional confounding variables (Yue Wu et al., 2024).

Attempts to acquire 2D NMR spectra on the same stored samples at -20 °C 12 months after initial 1D data acquisition were unsuccessful due to shimming issues and spectral degradation observed during post-thaw analysis. Visual inspection revealed crystalline precipitates in several buffered urine samples stored at −20 °C, consistent with previously reported calcium salt precipitation during cold storage (Gomez, 1995; Saetun et al., 2009). These crystals likely contributed to field inhomogeneities and loss of spectral coherence, which impaired 2D acquisition and limited our ability to annotate features beyond 1D spectra.

5.3 Data Analysis

Overview of Aims

Our analysis assessed the metabolic stability of voided canine urine samples stored under two common veterinary research conditions: refrigeration (4 °C) and room temperature (20 °C). Samples were evaluated over 72-hours using one-dimensional proton nuclear magnetic resonance (1D ^{1}H NMR) spectroscopy. Analytical objectives were to determine how time and

temperature influenced metabolite preservation and identify features demonstrating instability under different storage conditions. This section details the data handling and modeling strategy, including analytical adaptations required to overcome alignment challenges.

5.3.2 Study Design Considerations

Metabolomics in dogs has inherent challenges due to inter-individual variability. Unlike controlled laboratory animal studies, dogs may differ in breed, diet, lifestyle, and environmental exposures, which contribute to metabolic heterogeneity. These differences can confound biological interpretation if not adequately addressed during data analysis. In our study, exploratory principal component analysis (PCA) and hierarchical clustering revealed inter-dog variation exceeded variations associated with time or storage temperature. This finding indicated that the magnitude of individual metabolic differences could mask subtle stability effects, even under tightly controlled storage conditions. To address this, our analysis focused on methods reducing individual variability while also retaining stability-relevant information. This included performing alignment within each dog prior to attempting cross-dog comparisons and subsequently filtering for features that were consistently detected and well-aligned across all individuals in the final dataset. While this approach limited the total number of features retained for analysis, it increased confidence that the reported stability trends reflected true metabolite behavior rather than technical or biological noise.

5.3.3 Data Processing

NMR spectral data were initially processed using MNOVA (v14.2, Mestrelab Research), but phasing inconsistencies observed during quality control required transition to a batch-processing workflow using NMRPipe via the NMRbox platform. This approach provided improved baseline correction, alignment, and spectral uniformity. Reference deconvolution was applied to address

remaining shimming issues without re-acquiring the data. Processed spectra were subsequently analyzed using SAND. The original MNOVA-based output was excluded due to quality concerns, and all SAND analyses were re-run on NMRPipe-processed spectra.

Following SAND, feature alignment was conducted in a three-stage process: first within each individual dog, then across dogs, and finally filtered to retain only peaks consistently aligned across all six dogs. This alignment strategy was selected based on initial PCA and hierarchical clustering, which demonstrated that inter-dog variability exceeded that of time or storage condition. Ten dogs were excluded from the final analysis: eight formed a separate cluster and aligned well with each other but not with the main group, suggesting distinct inter-dog variation; the remaining two exhibited inconsistent spectral profiles that could not be reliably aligned. Data were then normalized using probabilistic quotient normalization (PQN) and mean-centered to correct for concentration differences and allow between-sample comparisons.

Several alternate preprocessing strategies, including variance plots, difference plots, and multi-rack normalization were evaluated but excluded due to poor performance or incompatibility with alignment consistency. A summary of all analytical approaches evaluated, and inclusion status is provided in Table 5.1.

To account for inter-individual variability, peak alignment was conducted using a three-stage process. First, peaks were aligned across all time points within each dog to control for subject-specific baseline variation which minimized technical noise and intra-individual drift unrelated to storage conditions. Second, aligned datasets from each dog were aligned together. Finally, features remaining consistently aligned and detectable across all six dogs were retained for analysis. This filtering step ensured that comparable features were used to assess stability.

Following this process, 107 aligned features were retained from an estimated 438 peaks collectively detected across all samples by the peak-picking algorithm, representing 24.4% of the total feature space.

Table 5.1 Summary of Analytical Strategies Evaluated During Data Processing and Analysis

Task	Final Inclusion	Purpose	Outcome & Rationale for Inclusion or Exclusion
Variance Plot	No	Identifies features with high variability across time points relative to baseline.	Excluded due to poor alignment across samples; variance plots require tightly aligned peaks.
Difference Plot	No	Computes the magnitude of change between baseline and later time points.	Requires perfect alignment.
MNOVA Pre-processing	No	Phasing and baseline correction using MNOVA software.	Replaced by NMRPipe batch processing.
Original SAND Pipeline	No	SAND used on NMR Pipe-Processed Data.	Shimming issues were identified in original SAND data, which affected downstream analysis; SAND re-run after reference deconvolution was employed
NMRPipe Batch Processing	Yes	Automated batch pre-processing (phase correction, baseline correction, etc.).	Used as the basis for all downstream steps.
Reference Deconvolution	Yes	Corrects for shimming differences without re-running samples.	Helped improve peak resolution. Final processing included re-SAND after deconvolution.
Unsupervised Clustering	Yes	PCA and hierarchical clustering to identify sample similarity and guide inclusion/exclusion.	Used to select dogs with the most comparable spectral profiles.
Processing and Transformation	Yes	Peak alignment by dog, followed by scaling and normalization.	Retained ~25% of original features after selecting consistently aligned peaks across all dogs.
Percent Change Threshold	Yes	Feature flagged as unstable if deviated >30% from baseline.	Primary method used to quantify temporal and condition-related instability.
Hypothesis Testing (T-test)	Yes	Paired comparisons of each time point to baseline and between storage conditions.	Identified features with statistically significant changes in intensity.
Mixed Effects Modeling	Yes	Modeled feature intensity across time and condition while accounting for repeated measures per dog.	Detected features with time, condition, and interaction effects, complementing t-test results.

5.3.5 Feature Stability Metrics

To evaluate temporal and temperature-related stability, we assessed changes in peak intensity for each of the 107 retained features across all time points and storage conditions. Stability was defined using a percent-change threshold approach: for each dog and feature, intensity values at subsequent time points were expressed as a percent change relative to the 0-hour baseline. A feature was considered unstable if its intensity deviated by more than 30% from baseline at any time point. This threshold was selected based on precedent in metabolomics stability studies and reflects a conservative estimate of biological and technical variability.

Statistical testing was performed to further evaluate temporal and temperature effects on metabolite intensities. For each feature, paired t-tests were used to compare post-baseline time points to the 0-hour measurement within each storage condition. Additional t-tests were conducted to compare corresponding time points between storage temperatures (4 °C vs. 20 °C). To account for repeated measures within individual dogs and assess interactions between time and temperature, mixed-effects linear modeling was used. In these models, time, temperature, and their interaction were treated as fixed effects, while individual dog was included as a random effect. This combined approach allowed us to assess both the magnitude and statistical significance of metabolite changes over time while accounting for subject-level variability. Features meeting either the percent-change instability threshold or showing significant differences across conditions were flagged for further review in downstream analysis.

5.4 Results

Sample Inclusion & Alignment Outcomes

Of the 16 dogs enrolled, 6 were retained for final analysis. Ten dogs were excluded following spectral processing: eight clustered separately and aligned internally but not with the main group,

and two displayed inconsistent spectral profiles. This prevented consistent feature alignment across individuals. Exclusions were based on assessments using PCA (Figure 5.1), hierarchical clustering (Figure 5.1), and manual inspection of alignment outputs. The final dataset consisted of 320 urine samples (6 dogs × 10 time points × 2 storage conditions, plus baseline aliquots), all processed using 1D ^{1}H NMR. Following the three-stage alignment process described above, 107 features were retained from the original 438 detected peaks (24.4%). This reduced set included features consistently aligned and present across all dogs and time points, minimizing spurious variability and enhancing interpretability of downstream analysis.

5.4.2 Stability Across Time & Temperature

Each retained feature was evaluated for relative stability using a 30% change threshold compared to baseline values. Stability counts at each time point under each storage condition are summarized in Table 5.2. Overall, samples stored at 4 °C exhibited greater feature stability than those stored at 20 °C, particularly beyond 6 hours. At 4 °C, most features remained within the 30% threshold up to 24 hours, while at 20 °C, deviations exceeding the threshold became common by 6 to 12 hours. These trends are visualized in Figure 5.3, which plots the percent of stable features over time point by condition.

To assess whether instability patterns were consistent across dogs, we identified features that exceeded the instability threshold in ≥50% of dogs at any time point. These are listed in Table 5.3, highlighting features most frequently unstable across individuals, especially under 20 °C storage conditions.

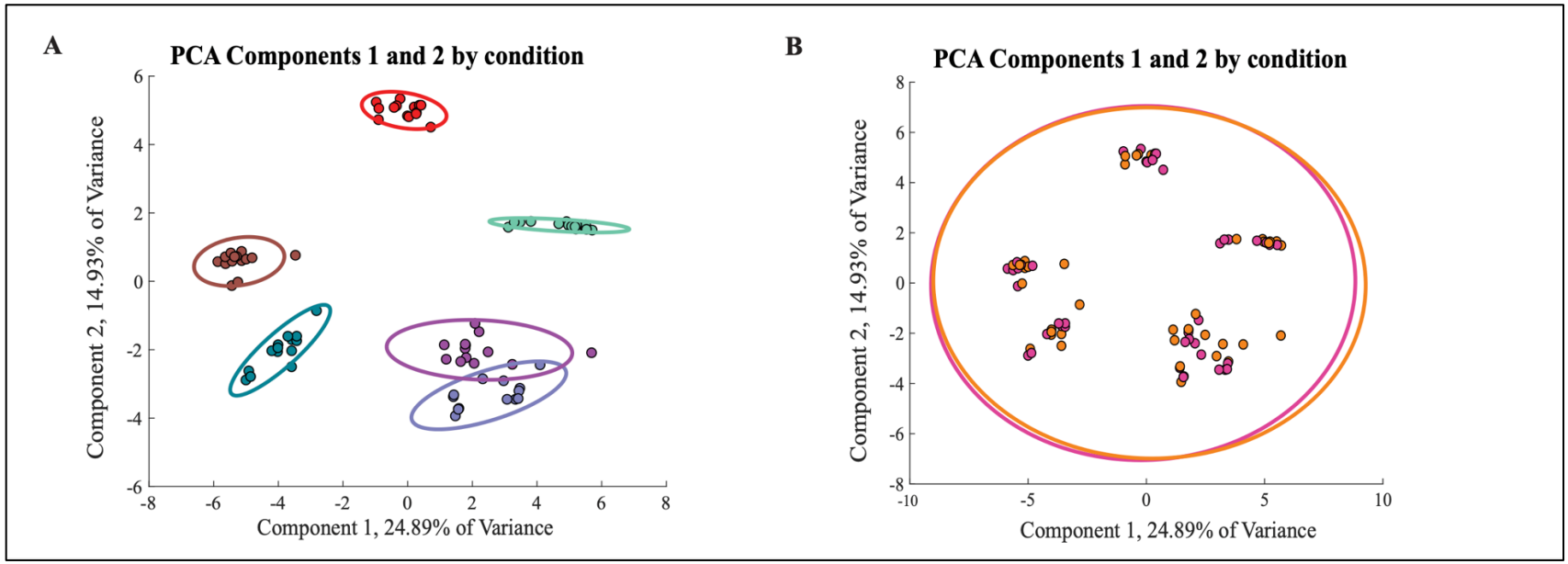

Figure 5.1. Principal Component Analysis of Aligned Metabolite Features by Dog and Storage Condition: (A) PCA of aligned features colored by individual dog. Each cluster represents repeated measurements from a single dog across time and temperature conditions. Strong separation between dogs and tight within-dog clustering supports the use of within-subject alignment prior to across-subject comparison. (B) PCA colored by storage condition (4 °C vs. 20 °C). Overlapping ellipses indicate lower between-condition variability compared to between-subject variability, reinforcing the dominance of inter-dog differences in the dataset

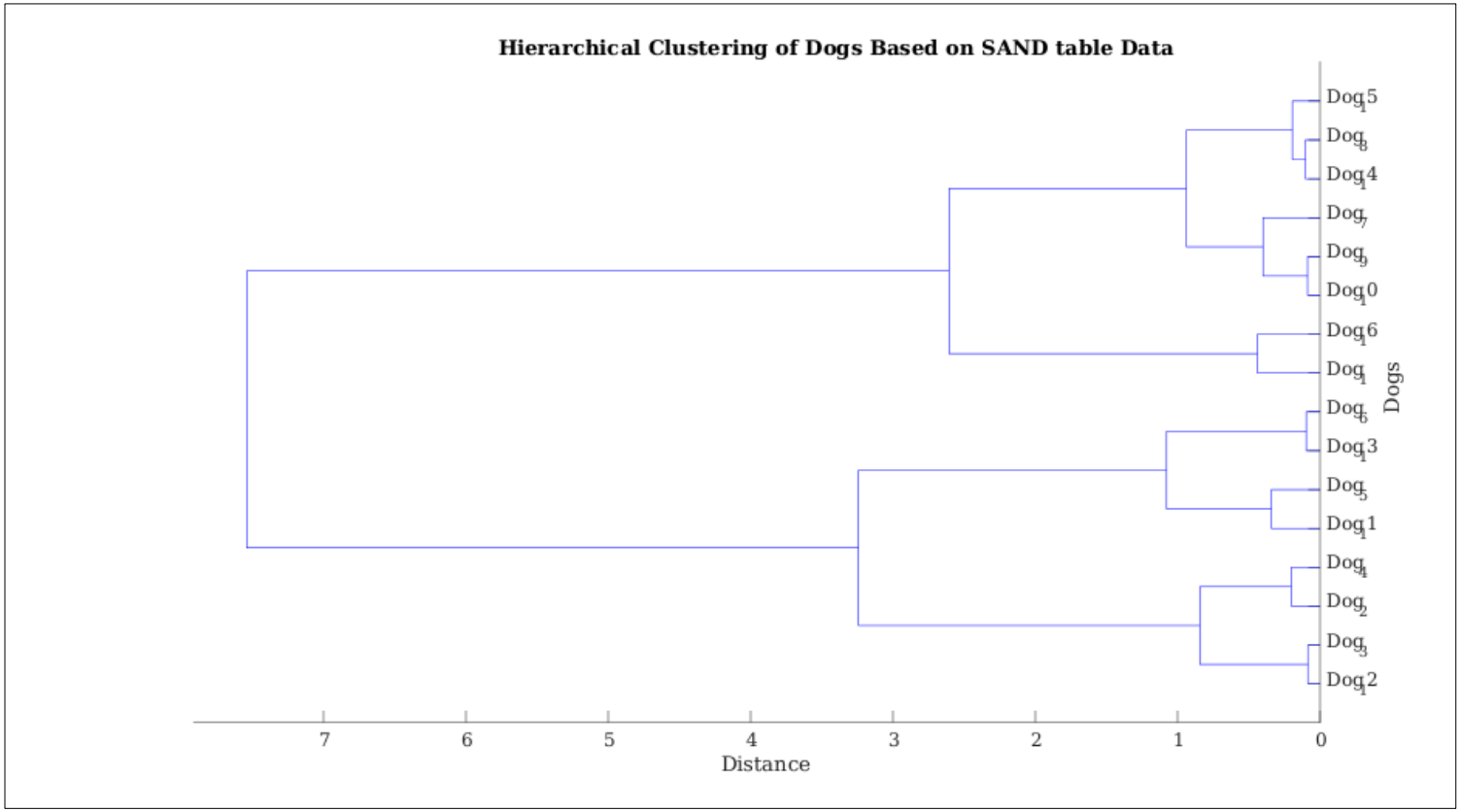

Figure 5.2. Hierarchical Clustering of Dogs Based on SAND Table Data. Dendrogram illustrating clustering patterns among all 16 dogs. Dogs retained for final analysis clustered together (bottom right), whereas 8 dogs formed a distinct upper branch, indicating consistent internal alignment but lack of comparability with the main group. Two additional dogs exhibited inconsistent profiles and poor integration with either cluster, supporting their exclusion.

Table 5.2 Feature Stability Summary Based on a 30% Change Threshold. Number and percentage of stable features at each time point under 4 °C and 20 °C storage. Stability defined as <30% deviation from baseline. 4 °C samples maintained >76% stability across all time points; 20 °C samples dropped to 58.9% by 72 hr, with most degradation occurring after 24 hr.

Feature Stability Summary					
Based on a 30% Change Threshold					
Condition	**Timepoint (hr)**	**Total Features**	**Stable**	**Unstable**	**% Stable**
4C	3	107	92	15	86
4C	6	107	88	19	82.2
4C	12	107	82	25	76.6
4C	24	107	85	22	79.4
4C	48	107	84	23	78.5
4C	72	107	86	21	80.4
20C	3	107	90	17	84.1
20C	6	107	90	17	84.1
20C	12	107	89	18	83.2
20C	24	107	90	17	84.1
20C	48	107	74	33	69.2
20C	72	107	63	44	58.9

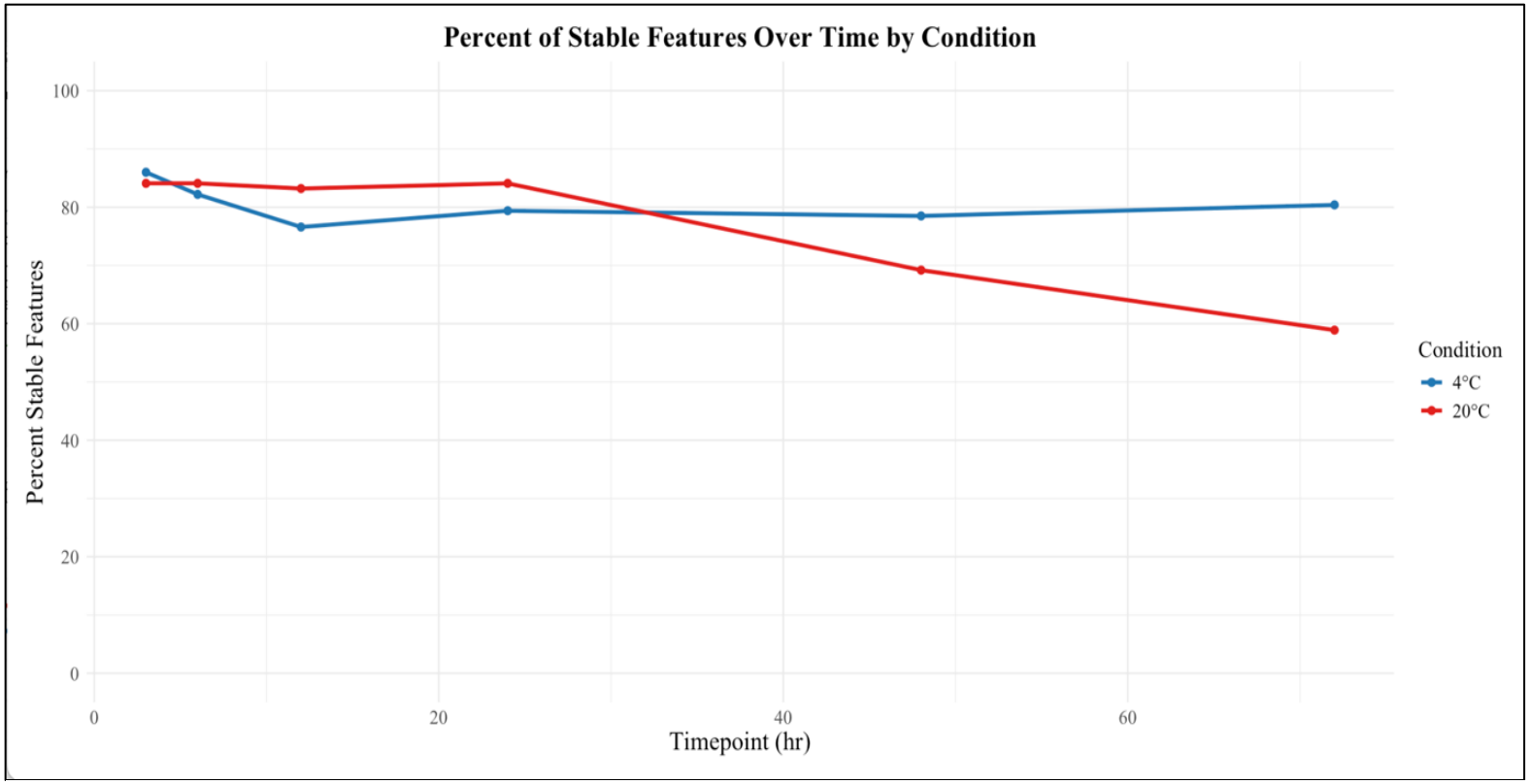

Figure 5.3. Features presented in Table 5.2 across the 72-hour study period. Trend demonstrates sustained metabolite stability in samples stored at 4 °C, with modest declines over time. In contrast, samples stored at 20 °C show accelerated loss of stability beyond 24 hours.

Table 5.3 Features exceeding 30% threshold. Features exceeding the 30% threshold in at least half of dogs at one or more time points. Most occurred under 20 °C storage, especially at 48 and 72 hr, indicating consistent temperature-sensitive behavior across individuals.

Features Unstable in ≥ 50% of Dogs					
Storage Condition	**Timepoint**	**Feature (Pietzsch & Hoppmann)**	**Dogs Unstable**	**Total Dogs**	**% Dogs Unstable**
20°C	72	4.516	5	6	83.3
20°C	6	4.516	4	6	66.7
20°C	24	4.516	4	6	66.7
20°C	48	4.6546	4	6	66.7
20°C	72	2.0028	4	6	66.7
4°C	3	1.6438	3	5	60
4°C	12	1.6438	3	6	50
4°C	24	4.516	3	6	50
4°C	48	4.516	3	6	50
4°C	72	2.0028	3	6	50
4°C	72	4.516	3	6	50
20°C	3	4.516	3	6	50
20°C	3	7.3809	3	6	50
20°C	12	4.516	3	6	50
20°C	48	1.9164	3	6	50
20°C	48	2.0028	3	6	50
20°C	48	2.0961	3	6	50
20°C	48	2.2654	3	6	50
20°C	48	4.516	3	6	50
20°C	72	1.9164	3	6	50
20°C	72	4.6413	3	6	50
20°C	72	4.6546	3	6	50

Statistical testing using paired t-tests identified limited but notable deviations from baseline at 20 °C compared to 4 °C. Only one feature exceeded significance thresholds by percent change and paired testing, and two by mixed-effects modeling. These findings suggest that while most features remained stable, a small subset may be particularly susceptible to degradation at room temperature over time. Differences between temperatures at each time point were limited in statistical significance, with only a small number of features primarily after 12 hours exceeding significance thresholds. Mixed-effects modeling confirmed significant main effects of time and temperature, as well as interaction effects for a subset of features, reinforcing the observed trend that prolonged exposure to room temperature accelerates metabolic degradation or transformation.

5.4.3 Time of First Instability

To further evaluate degradation patterns, we examined when each feature first exceeded the 30% deviation threshold. This provided insight into timing and progression of instability under each condition. Table 5.4 summarizes the number of newly unstable features at each time point and reports median, earliest, and latest onset. Compared to 4 °C, features stored at 20 °C became unstable later but in greater numbers. Figure 5.4 visualizes these trends. At 4 °C, instability began earlier (median: 6 hr) but progressed gradually, while at 20 °C, instability onset was more delayed (median: 24 hr) but associated with a higher total number of unstable features by the end of the study period.

5.4.4 Mixed-Effects Modeling and Feature-Specific Trends

Mixed-effects models were used to account for repeated measures and assess the effects of time, temperature, and their interaction on feature stability. Two features showed significant change over time, one feature differed consistently between storage conditions, and twelve features

demonstrated a significant time × temperature interaction indicating condition-specific instability patterns.

To explore the most frequently unstable features, the top 25 unstable features under each condition were identified based on the number of instability events across dogs and time points (Figure 5.5). A total of 43 unique features were unstable at 4 °C, and 59 at 20 °C. While many features repeatedly exceeded the 30% threshold, notably at 20 °C, few met statistical significance criteria, suggesting broad feature-level instability may not always result in statistically distinct degradation trajectories.

5.4.5 Summary of Analytical Workflow

The final workflow, previously summarized in Table 5.1, prioritized reproducibility and conservative feature selection. Computational refinement remains ongoing to improve the inclusion of excluded dogs in future analyses of this work.

Table 5.4 (A/B) Timing of Feature Instability by Storage Condition: (A) Summary of when features first exceeded the 30% deviation threshold. “Newly Unstable Features” reflects first-time instability at each time point. Lower panel (B) shows median, earliest, and latest onset. Instability occurred earlier and more frequently at 20 °C (median 6 hr, 59 features) vs. 4 °C (median 24 hr, 43 features), supporting refrigeration for short-term preservation.

(A) Feature Instability Counts		
Condition	**First Unstable Time**	**Newly Unstable Features**
4 °C	3	15
4 °C	6	11
4 °C	12	10
4 °C	24	1
4 °C	48	5
4 °C	72	1
20 °C	3	17
20 °C	6	7
20 °C	12	5
20 °C	24	6
20 °C	48	14
20 °C	72	10

(B) First Instability Median				
Condition	**Median First Instability**	**Earliest Time**	**Latest Time**	**Total Unstable Features**
4 °C	6	3	72	43
20 °C	24	3	72	59

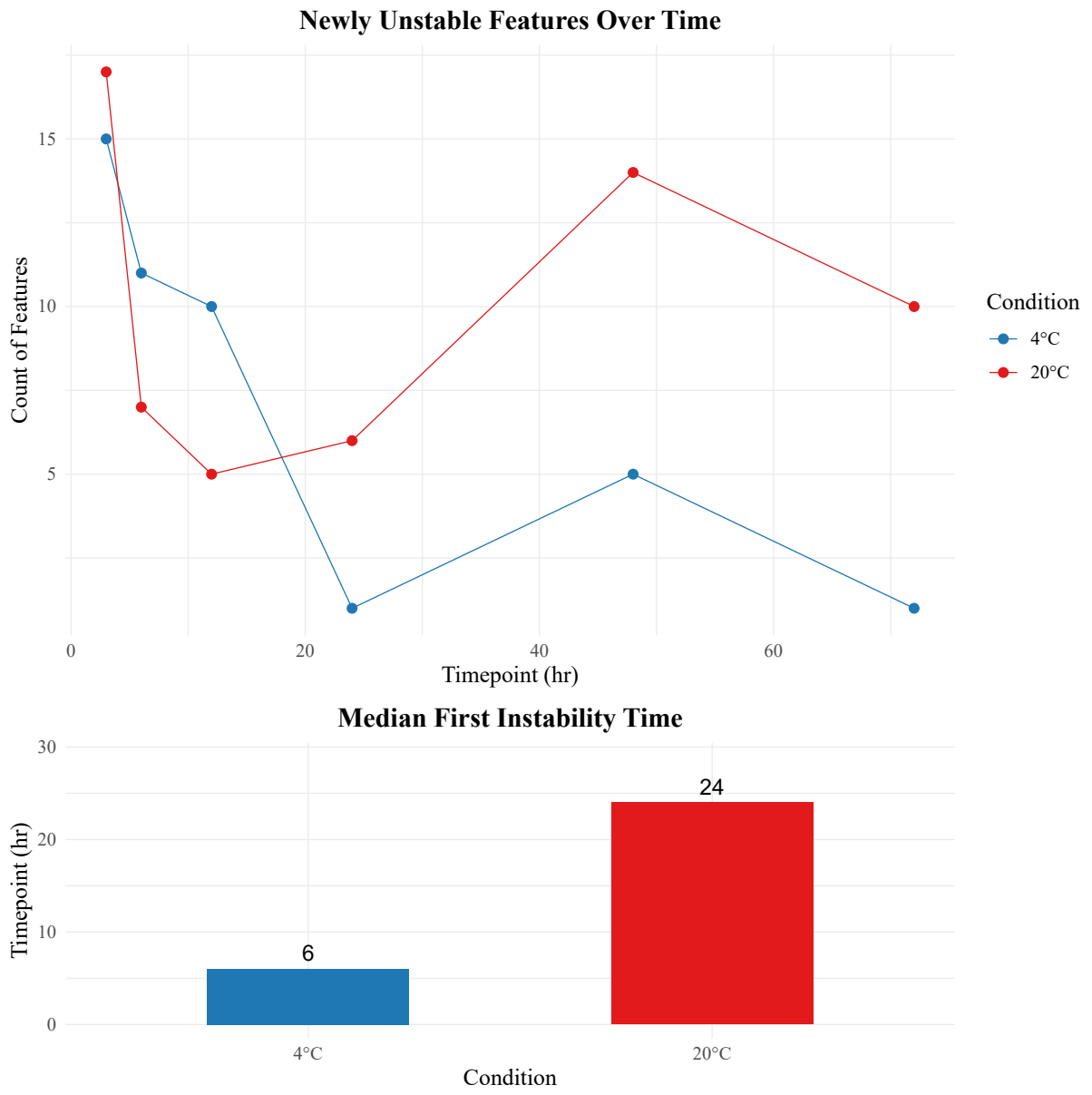

Figure 5.4 (A/B) Onset & Progression of Feature Instability by Storage Condition: (A) Number of features exceeding the 30% instability threshold for the first time at each time point. Instability begins earlier at 4 °C but affects fewer features overall. At 20 °C, onset is delayed but leads to a greater number of unstable features, particularly after 24 hours. (B) Median first-time instability for all unstable features under each storage condition. Median onset occurred at 6 hours for 4 °C & 24 hours for 20 °C, supporting that refrigeration delays metabolite degradation.

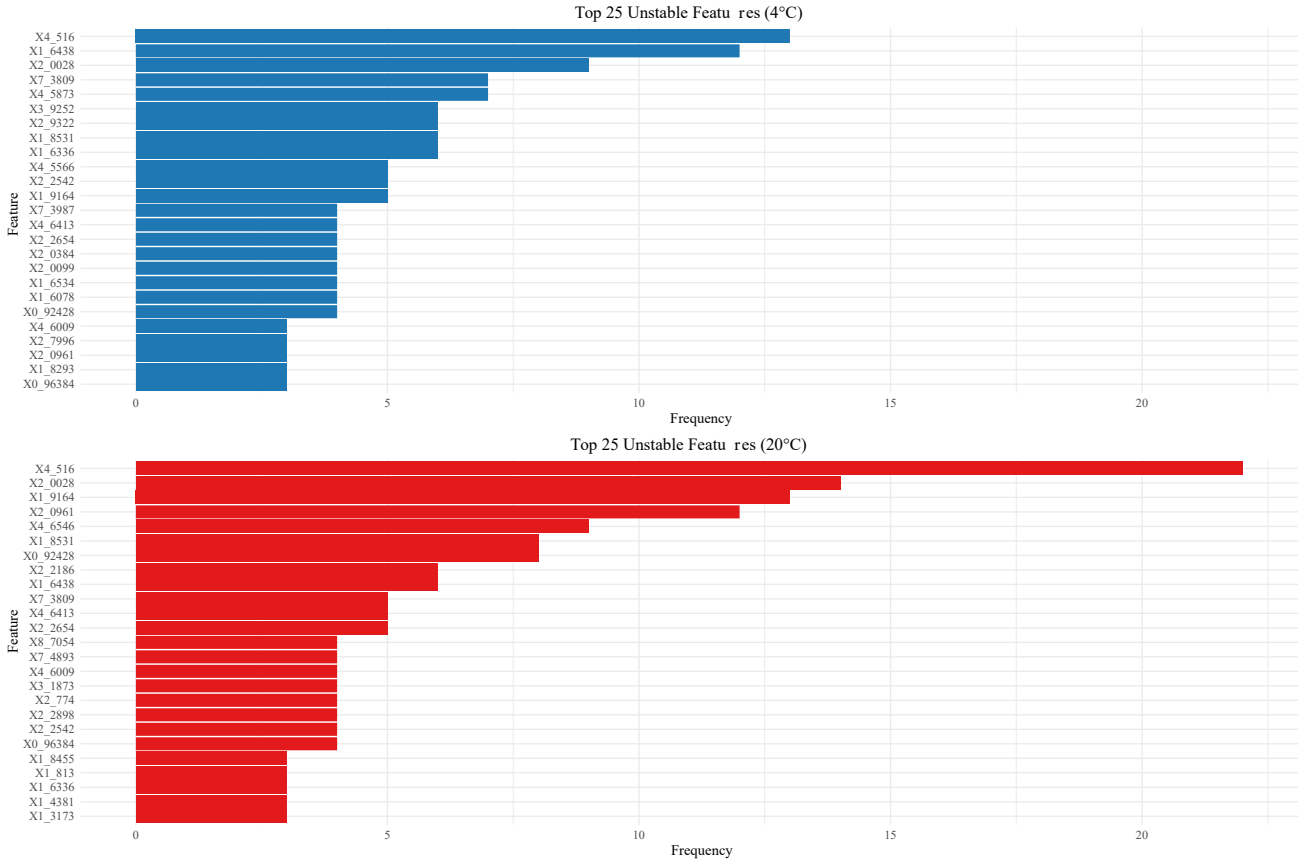

Figure 5.5 Top 25 Unstable Features by Storage Condition & Frequency: Bar plots show the top 25 features exhibiting instability (≥30% change from baseline) most frequently across dogs and time points under 4 °C (top) and 20 °C (bottom) storage. Bar length reflects the number of instability occurrences per feature. A total of 43 unique features were unstable at 4 °C, and 59 at 20 °C. Only one feature reached significance by t-test and two by mixed-effects modeling, suggesting that while many features show repeated instability, few exceed statistical thresholds, especially at 4 °C. Instability is more widespread and recurrent at 20 °C.

5.5 Discussion

This study evaluated the stability of canine urine samples for metabolomics investigations under two common storage conditions using untargeted 1D ^{1}H NMR. While inter-individual variability and technical factors (e.g., spectral alignment, post-thaw crystallization) influenced the final dataset, these challenges informed methodological decisions, resulting in a high-confidence subset of 107 consistently aligned features across six dogs. The focused dataset enabled robust assessment of pre-analytical stability under conditions relevant to veterinary and translational research.

Samples stored at 4 °C retained a higher proportion of stable features across time points, while samples stored at 20 °C exhibited a sharper decline in stability after 24 hours. Although 4 °C appears to preserve stability more effectively, this conclusion is based on a limited group of features from a larger dataset and lacks technical replicates. Future studies should build on this work with more controlled designs and replicate sampling to improve generalizability.

The inability to capture 2D NMR spectra from stored samples highlights an often-overlooked limitation in metabolomics: the assumption that prepared, buffered urine remains analytically stable during frozen storage. While raw urine stored at -80 °C or -20 °C has shown long-term stability, this does not extend to samples prepared for NMR analysis. In our study, attempts to acquire 2D data one year post-preparation failed due to precipitation and crystallization in thawed samples, likely calcium- or phosphate-based, that disrupted shimming and spectral coherence (Gomez, 1995; Saetun et al., 2009). While sodium azide effectively inhibits microbial activity, it cannot prevent physical matrix changes such as precipitation. These findings underscore the need for careful storage planning when high-resolution NMR analysis is intended.

The protocols applied in this study were based on established human urine metabolomics methodologies (Dona et al., 2014), which were optimized and intended for large-scale, high-throughput analyses. These protocols ensured post-collection uniformity and throughput. However, the protocol did not include controls for pre-analytical variation during urine collection. Additionally, we incorporated updated recommendations for minimizing cellular disruption through gentle centrifugation (Emwas et al., 2015; Patrizia et al., 2011). These investigated the influence of initial collection and sample handling regarding minimization of cellular disruption. While our sample preparation methods aligned with the best practices available at the time of protocol adoption, future studies involving dog urine where cellular and microbial variability is higher, would benefit from incorporating updated collection and centrifugation practices to further preserve the biological integrity of the metabolome.

In addition, our findings emphasize the high degree of individual variability among dogs, a reflection of real-world clinical diversity. This variation aligns with expectations for companion animal studies and suggests targeted analytical strategies may be more practical and interpretable in clinical or translational research settings.

This study highlights the importance of appropriate statistical models and experimental design in untargeted metabolomics, particularly when applied to veterinary populations. While our scope was narrowed by sample storage post 1D data acquisition and alignment constraints, outcomes provide guidance toward improving methodological standards and reproducibility in canine metabolomics. These considerations are expanded further in the next chapter, which outlines evidence-based recommendations for sample handling, storage, and study design.

Footnotes

a - Animal Care and Use Approval:
This protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia in compliance with the Animal Welfare Act.

CHAPTER 6

RECOMMENDATONS FOR HANDLING DOG URINE SAMPLES IN METABOLOMICS STUDIES: INTEGRATION OF VETERINARY AND HUMAN PRACTICE

[1]Nicole R. Cammack, Joseph W. Bartges. To be submitted to a peer-reviewed journal.

Abstract

Metabolomics is a valuable tool for advancing canine health research, although methodological inconsistencies in dog urine sample handling limit reproducibility and cross-study comparisons. Human studies benefit from standardized protocols, however veterinary applications lack uniform guidance for urine collection, processing, and storage. This chapter integrates current literature and findings from prior investigations to provide recommendations for sample collection, pre-analytical handling and storage of dog urine in metabolomics studies. Aligning veterinary protocols with established human metabolomics standards, while accounting for species-specific factors, is imperative for high data quality and enabling reliable biomarker discovery. These recommendations support more consistent, reproducible, and interpretable urine metabolomics research in dogs, with relevance to veterinary medicine, sample biobanking, nutrition, and comparative research initiatives

6.1 Introduction

Metabolomics is increasingly recognized as a powerful tool for exploring dogs' health, disease, and physiological processes in veterinary medicine. Urine, a non-invasive and readily available biofluid, is well suited for metabolomics studies due to its metabolite richness and ease of collection (Bouatra et al., 2013; A. Zhang et al., 2012). In human metabolomics, decades of research have resulted in detailed urine sample collection, handling, storage, and preparation protocols that have enhanced the quality and reproducibility of research in the field (Bouatra et al., 2013; Raúl González-Domínguez, 2020; Wishart, 2019). Additional work has detailed the importance of fully documenting methodology in metabolomics studies for analytical quality and reproducibility (Kirwan et al., 2022). However, in veterinary context, particularly in dog urine metabolomics, absence of standardized protocols has led to inconsistencies across studies, potentially compromising the reliability and comparability of results.

Given growing interest in metabolomics technologies for investigating canine health, and for use in comparative research, there is demonstrated need to establish standardized protocols for dog urine sample handling which align with best practices from human metabolomics. This work addresses this gap by providing a rationale for standardized sample collection, handling, and storage practices. The first objective is to establish the foundational rationale of pre-analytical variables that influence data quality and reproducibility as informed existing practices in canine urine metabolomics, integrating findings from our AGE and urine stability investigations presented in earlier chapters, and integration of relevant human protocols. The second is to offer step-by-step pre-analytical sample collection, handling, and storage recommendations informed from the first objective. While specific protocols for sample preparation and platform-specific methodologies for mass spectrometry (MS) or nuclear

magnetic resonance (NMR) spectroscopy are beyond the scope of this chapter, the pre-analytical recommendations outlined here are focused on NMR but are applicable across both analytical approaches.

6.2 Consideration of Pre-Analytical Factors in Dog Urine Metabolomics Studies

Avoiding unintended bias is an important factor for any metabolomics investigation (Barnes et al., 2016). Inter-individual variation in breed, age, diet, activity level, sex, neuter status, and environment can influence the metabolome and obscure group-level patterns (Abdul-Hamid et al., 2015; Beckmann et al., 2010; Jianqiang Wu, 2015; Lau et al., 2018). Narrower inclusion criteria (e.g., single breed, standardized diet or environment) or within-subject study designs may reduce noise and improve interpretability.

6.2.2 Standardization of sample collection

Standardize urine collection methods across all subjects to minimize variability, whether using free-catch, cystocentesis, or catheterization. Urine collection should be planned at consistent times, (e.g. first-morning void) as composition varies throughout the day (Dallmann et al., 2012). While differences between first-void and mid-stream urine are minimal (Giskeødegård et al., 2019), mid-stream catch should be prioritized for canine studies due to potential risk of bacterial contamination. Once collected, all samples should be stored on ice and during subsequent processing prior to storage.

6.2.3 Minimization of external variables

Many external variables can influence the data quality and subsequent analyses. These differences can lead to spurious differences between groups leading to mis-categorization of biologically relevant changes (Barnes et al., 2016). Dietary control (e.g. diet format, nutrient composition, calorie intake) of study subjects before and during urine collection is important

since diet has been demonstrated to be a major factor influencing metabolite profiles (Giskeødegård et al., 2019; Robertson et al., 2011). Standardization reduces potential confounding effects. Consider controlling or recording physical activity and environmental conditions, as these have been shown to influence the metabolome (Emwas et al., 2015; Jianqiang Wu, 2015). Medications and supplement intake should be considered. Diuretics, antidiuretics, and nephrotoxicants alter urine salt concentration, pH, and protein content (Schreier et al., 2013). If these factors are unaccounted for during preparation, they can affect metabolite detection and introduce challenges for data analysis.

6.2.4 Collection Containers

High-quality inert collection containers minimize contamination and chemical interactions with urine components (Yao et al., 2016). Polypropylene is recommended due to chemical stability and low reactivity. The same type and manufacturer of containers should be used across all samples within a study to minimize variability between container types. Variations introduce confounding variables, such as different rates of chemical leaching or contamination (Raúl González-Domínguez, 2020; Rist et al., 2013; Yao et al., 2016). Collected samples should be kept on ice after collection and during subsequent aliquoting, centrifugation, and associated steps. The time between sample collection and placement on ice should be documented.

6.2.5 Aliquot containers

There is evidence to suggest the type of collection container can influence metabolomic profiles of samples, and the same is true for containers used during sample processing (e.g. aliquot or centrifugation tubes). Therefore, it is important to document type, manufacturer, and material of all containers used for urine collection, transfer, and storage. Like collection containers, this is important for reproducibility of the study and enabling comparisons across cohorts, labs, and

studies. Polypropylene is also recommended for aliquot containers recommended due to low reactivity and permeability to CO_2, which could alter sample pH (Yao et al., 2016). Notably, polypropylene has been found permeable to CO_2 when frozen on dry ice (Rist et al., 2013). However, -20°C and -80°C did not demonstrate CO_2 permeability (Rist et al., 2013). If freezing samples on dry ice, the use of borosilicate glass tubes could be considered (Rist et al., 2013). Researchers should minimize urine transfer between containers, and when transfer is necessary, ensure that receiving containers are the same material and quality as the original collection container.

6.2.6 Aliquoting

After urine collection, aliquot samples into polypropylene cryogenic vials, ideally that fit in a centrifuge to minimize sample transfer. Preparation of multiple smaller aliquots rather than one large aliquot minimized the time samples are subjected to ambient temperature and limits freeze-thaw cycles (Want et al., 2010).

6.2.7 Considerations for Cell and Enzyme Content of Sample

Cellular debris and intact cells lyse upon freezing and release enzymes or metabolites, that alter the urine metabolome and cause pH shifts. A study demonstrated that urine samples not pre-centrifuged prior to storage had greater variance in metabolomic profiles after freezing at -80 °C than those flash-frozen in liquid nitrogen, likely attributable to lysing at slower freeze (Patrizia et al., 2011). Although these shifts are smaller than typical biological variation, they introduce unavoidable artifacts.

6.2.8 Centrifugation

Once aliquoted, samples should be gently centrifuged (1,000-3,000 RCF) for 5 minutes at 4°C, if not being flash frozen via liquid nitrogen. This process removes cellular debris and other

particulates that affect the metabolome and pH without inducing cell lysis (Patrizia et al., 2011). Higher RCFs can lyse leading to cellular components inducing pH changes, and should be avoided. If centrifugation is skipped, lysing also occurs upon freezing unless frozen in liquid nitrogen (Patrizia et al., 2011). The amount of changes are directly related to the amount of cells present, with higher amounts inducing greater pH changes than little to no cellular components. The pH change, while not directly responsible for new peaks or peak changes, does induce chemical shift changes that complicate analyses (Patrizia et al., 2011). Centrifugation should be conducted at 4 °C, as temperature differences influence results (Ammerlaan et al., 2014). Ensure documentation of centrifuge brand, model, rotor diameter and centrifugation settings.

6.2.9 Filtration

Optional filtration (0.2 μm filtered pipette tip) may be performed, based on study objectives and considerations regarding collection methods, to remove bacteria and remaining debris. It has been reported that filtration with centrifugation, is the most effective method for removing bacteria and cellular debris in urine and has been previously recommended (Patrizia et al., 2011; Saude & Sykes, 2007). If filtration is used, document filter type, and manufacturer.

Filtration can be considered for samples subjected to storage delay before -80°C. Although, if aliquoting and centrifugation immediately follow collection and samples are stored at -80°C following supernatant transfer to a fresh cryogenic vial, filtration may not be necessary. While filtration reduces bacterial contamination, it may remove some proteins or introduce artifacts (Raúl et al., 2020). Thus, consider use based on study objectives.

6.2.10 Preservatives

Chemical preservatives (e.g. sodium fluoride (NaF), sodium azide (NaN_3)) are generally not recommended in urine metabolomics studies if samples are stored appropriately (Lauridsen et al.,

2007). Although preservatives inhibit bacterial growth, they induce ion-binding activities interfering with detection and quantification of metabolites (Aziz et al., 2021; Lauridsen et al., 2007). NaF, for example, induces metabolome changes due to its ion binding ability, potentially leading to inaccurate results. Similarly, NaN_3, while effective at preventing bacterial contamination, is unnecessary when proper centrifugation and freezing protocols are followed. Immediate sample processing (e.g. centrifugation and/or filtration), and -80 °C freezing is advised to maintain sample integrity without introducing chemical artifacts (Patrizia et al., 2011). Rather, addition of preservatives is recommended in the sample preparation phase to prevent microbial growth during data acquisition (Lauridsen et al., 2007; Saude & Sykes, 2007).

6.2.11 Storage Considerations

Current metabolomics guidelines emphasize consistency in biobanking practices to preserve sample integrity. Several studies have demonstrated importance of immediate freezing at either -20 °C or -80 °C to preserve the metabolite profile (Patrizia et al., 2011; Saude & Sykes, 2007). However, other work has demonstrated viability of samples stored at 4 °C for up to 24 hours (Dunn et al., 2008). Each of these storage methods have considerations for dog urine metabolomics.

6.2.11.2 Short-Term Storage: Less than 6 Months

Urine samples may be stored at 4 °C for short durations for at least 24 hours, particularly when immediate freezing at -20 °C or -80° C is not feasible. One study showed human urine is stable up to 48 hours at 4 °C (Gika et al., 2008), however further validation of this method would be advisable. More specifically, samples stored in these ways are likely stable if collected on ice, gently centrifuged (1,000-3,000 RCF) to remove cells and debris as described above (Aurelie et al., 2015; Budde et al., 2016; Dunn et al., 2008; Gika et al., 2008; Patrizia et al., 2011). Short-

term storage allows for logistical flexibility, especially in home collection or transport to a processing facility. However, urine stability in human samples when stored beyond this time frame has been shown to be unreliable (Saude & Sykes, 2007). Therefore, it is advisable to validate storage methods for short term storage methods, especially when not stored at -20 °C, -80 °C or subjected to pre-analytical processing and storage delays.

Short-term storage, up to 6 months, at -20°C is acceptable if -80°C is not immediately available, or if 4 °C storage will exceed 24 hours. Studies have demonstrated stability of urine metabolites at -20°C up to 6 months, with minimal impact on the metabolite profile with pre-storage centrifugation (1,000-3,000 RCF) (Gika et al., 2008; Laparre et al., 2017; Lauridsen et al., 2007; Stevens et al., 2019). Although consensus for short term storage of urine for use in metabolomics studies at -80 °C is preferred as it effectively "pauses" biochemical activity, so long as cellular debris are removed (Stevens et al., 2019).

6.2.11.3 Long-term Storage: More Than 6 Months

Long term storage of urine at -80 °C for 12 months is acceptable in metabolomics biobanking practice, although few studies beyond 6 months at -80 °C are available. Ghini et al. (2019) demonstrated that when mild centrifugation (1,000-3,000 RCF) and filtration (0.2 μm filtered pipette tip) are utilized to remove cellular and bacterial content, urine samples stored at -80 °C remain stable up to five years, with negligible changes (Ghini et al., 2019). This supports that deep-frozen, well-prepared urine samples are suitable for delayed analysis. In contrast, unprocessed urine exhibited pH drift, signal shifts, and metabolite degradation over time, demonstrating importance of standardized pre-analytical protocols to preserve reproducibility and biological interpretability of data from biobanked samples (Ghini et al., 2019). Other works have provided data demonstrating stability of specific metabolites, or metabolite classes, for

longer than 6 months at -70 °C (Mazumder et al., 2023; Samandar et al., 2009), to the authors knowledge there are limited additional studies investigating stability of urine subjected to long term storage at -80°C for untargeted metabolomics research.

Ideally urine samples should be centrifuged prior to storage regardless of conditions to mitigate the risk of pH changes and bacterial contamination. These mitigation practices reduce analytical challenges that arise from such changes.

6.2.11.4 Additional Storage Considerations

Alternatively, collected samples could be immediately aliquoted, skip centrifugation and filtration, and frozen via liquid nitrogen, or liquid nitrogen vapor. Samples stored this way are most stable compared to other methods as freezing below -130°C prevents crystallization and cell lysis, preserving sample integrity and cellular components (Patrizia et al., 2011). This method is most likely ideal for these reasons; however in most cases (e.g. larger cohorts) this may be cost-prohibitive and labor-intensive.

6.2.12 Thawing Procedures

Thaw aliquots over 1-2 hours at 4°C, or on ice, avoiding extended thawing periods, which compromises sample integrity (National Cancer Institute, 2016). Room temperature thawing should be avoided if possible, to minimize exposure to conditions that may compromise sample integrity.

6.2.13 Sample Preparation Before Data Acquisition

Methods for urine metabolomics sample preparation differ based on the platform and study objectives. Although sample preparation methods are outside the scope of these recommendations, given numerous platforms and methods used, authors should fully document

all sample preparation steps, including the reagents used (with manufacturer details), preparation protocols, and equipment settings (e.g. centrifugation speed, filtration, vortex conditions).

Limit the time between sample preparation and data acquisition to reduce the risk of metabolite degradation. Robotic sample handlers can be used in sample preparation, especially in large high through-put studies and have been detailed elsewhere (Anthony C. Dona, 2014). While robotic-assisted sample preparation eliminates human error, consider the time robotic-assisted sample preparation may take and ensure sample temperature integrity. Large studies may require batch preparation to limit time between thawing and analysis. Regardless, studies should report of robot manufacturer, time between thawing, preparation and analysis as well as temperature control of the samples.

6.2.14 Storage During Data Acquisition: Temperature Control

During data acquisition, if using automated systems or robotic handlers, ensure samples are kept at a controlled temperature (preferably 4°C). Prolonged exposure to room temperature or fluctuating conditions can alter metabolite profiles. Document the storage conditions and any delays between preparation and data acquisition.

Experimental details for data acquisition, are outside the scope of this work and have been detailed elsewhere (Tzoulaki et al., 2014). However, regardless of platform, detailed documentation of data acquisition parameters and experiments used are essential in published studies.

6.2.15 Reanalysis of Prepared Urine Samples for NMR: Stability and Crystallization

Post-Analytical Freezing & Sample Stability

In NMR-based metabolomics, urine is typically buffered with ~0.1 M KH_2PO_4 to stabilize pH and treated with preservatives (e.g. sodium azide) to inhibit microbial growth during analysis. It

is common for prepared samples to be stored at 4° C or frozen at -20 °C for reanalysis. However, challenges may arise for buffered samples that are frozen. One study demonstrated that up to 5-8 freeze-thaw cycles had negligible impact on ^{1}H NMR spectra of buffered rat urine (Schreier et al., 2013), indicating short-term refreezing is unlikely to compromise metabolite quantification. Additionally, samples stored at 4 °C for up to 14 days, or at -20 °C for up to 24 months showed no significant spectral changes in 1D 1H NMR. However, these findings are distinct to 1D analysis, and effects on 2D NMR resolution and reusability after long term storage remain unknown. In our dog urine stability pilot study, buffered urine samples stored at -20 °C for 12 months showed visible precipitate formation, with corresponding loss of 2D NMR spectral quality and spectral misalignment. These precipitates prohibited capture of 2D NMR experiments within our canine urine stability study which aligns with documented challenges in urine storage (Lauridsen et al., 2007).

6.2.19 Analytical Impact of Precipitate Formation

Precipitate formation and crystalline hydrates formation present analytical challenges. Sodium phosphate buffers can form hydrates (e.g. $Na_2HPO_4 \cdot 12H_2O$), altering monobasic/dibasic ratios and shift pH (Gomez, 1995). Urine is rich in salts (e.g. calcium, oxalate, phosphate, urea) that become supersaturated at low temperatures and result in precipitation and formation of inorganic crystals from buffer or biological solutes (Gomez, 1995). One study noted challenges in analyzing frozen urine samples due to formation of precipitates, subsequently identified as inorganic crystals, primarily calcium oxalate dihydrate along with other amorphous calcium salts (Saetun et al., 2009). In tests where urine was aliquoted, centrifuged to remove cellular debris, and frozen at -20 °C overnight, a visible sediment formed upon thawing (Saetun et al., 2009).

These precipitates compromise 2D NMR experiments which requires a homogenous solution for optimal spectral resolution. Like 1D 1H NMR, 2D acquisition is sensitive to magnetic field inhomogeneities from precipitate. Crystals introduce microheterogeneity, hinder magnetic shimming, and entrap low-molecular-weight analytes, leading to distorted peaks and chemical shift variability.

While a quick 1D ^{1}H NMR re-run on thawed samples may be highly similar to initial runs, 1D and 2D NMR re-runs on samples stored for longer periods of time could reveal consequences of these longer freeze-thaw cycles. We observed substantial spectral misalignment, loss of 2D spectral quality, and batch drift when analyzing samples stored at -20°C for several months within our dog urine stability study. This aligns with caution from a physiological study that freezer-induced precipitates must be considered when analyzing previously frozen urine (Saetun et al., 2009).

6.2.20 Mitigating Precipitation & Crystallization

To preserve reusability buffer-prepped samples for NMR, several strategies can be utilized. First, storage duration at -20 °C should be minimized, particularly when reanalysis is anticipated. Samples intended for repeat acquisition should ideally be reanalyzed within days or weeks rather than stored for months. Some researchers bypass need for refreezing altogether by storing samples at 4 °C and re-running within 24-48 hours, a practice supported by evidence properly handled urine remains stable when refrigerated for short periods (Aurelie et al., 2015). When feasible, study design should include creation of multiple aliquots from the original sample prior to analysis, allowing for future reanalysis without subjecting the sample to repeated freeze-thaw cycles.

In cases where aliquoting is not feasible, samples should be thawed (on ice) and visually inspected for evidence of precipitates. Vortexing may re-dissolve urea and some salts, while persistent precipitates may be removed by centrifugation, allowing supernatant recovery. However, removing precipitates may also remove analytes (e.g. Ca^{2+} and anions) may result in sample being no longer representative of the original composition. Such losses may be tolerable if they are not primary study targets, although depletion does introduce variability that may compromise data integrity. Lastly, all samples should be handled using consistent, well-documented procedures. Uniformity in freezing and thawing procedures is essential. For ensuring observed differences in metabolomic profiles reflect biological variation rather than pre-analytical artifacts (Rist et al., 2013).

6.3 Recommendations for Clinical and Experimental Urine Collection, Handling & Storage of Dog Urine for Metabolomics Investigations

Table 6.1 outlines recommendations are based on best practices for human and canine urine metabolomics and tailored to address specific challenges and offer practical substitutes for dog urine metabolomics investigations. These recommendations are a summary of information found in the previous section.

6.4 Conclusion

The detailed recommendations presented here aim to standardize the collection, handling, and storage of dog urine samples in metabolomics studies for the purpose of enhancing reproducibility and reliability of research outcomes in the field. While these guidelines focus on sample preparation steps, ensuring thorough documentation of data acquisition settings, equipment used, software, data processing, and analytical methods is equally vital. Given the complexity of metabolomics, supplementary information may be required to meet journal

publishing requirements, ensuring that all methodological details are transparent and reproducible.

Table 6.1. Pre-Analytical Recommendations for Dog Urine Metabolomics Studies.

Workflow Stage	Procedure Step	Recommended Best Practice	Practical Substitute	Context-Dependent Alternative
Study Design & Pre-Sample Collection	**Sample Size & Population**	Determine based on statistical power, cost, recruitment, & throughput. Use small cohorts for pilot studies.	N/A	N/A
	Controls	Use healthy dogs, defined nutritional & activity regimens.	Document differences & rationale.	N/A
	Collection Method	Standardize method (free-catch, cystocentesis, catheterization).	Document differences & rationale.	N/A
	Collection Timing	Collect at consistent times (e.g. first-morning void).	Document differences & rationale.	N/A
	Midstream vs First-Void	Use midstream catch to reduce contamination.	Document differences & rationale.	N/A
	Feeding Status	Standardize feeding status pre-collection.	Document differences & rationale.	N/A
	Diet Control	Consider control of diet format, nutrient composition, & caloric intake.	Document differences & rationale.	N/A
	Physical Activity & Environment	Standardize & document.	Document differences & rationale.	N/A
	Medications & Supplements	Document all medications & supplements.	N/A	N/A
	Container Types	Use polypropylene; standardize & document type, manufacturer, material.	N/A	N/A
Sample Handling	**Post-Collection Cooling**	Keep samples on ice post-collection & during processing.	N/A	N/A
	Container Transfer	Minimize transfers; use identical material to collection container.	N/A	N/A
	Aliquoting	Aliquot 1–2 mL into cryogenic vials ASAP post-collection, Prepare multiple aliquots to limit freeze–thaw.	N/A	N/A

	Centrifuge	1,000–3,000 RCF, 5 min at 4 °C. Document centrifuge brand, model, rotor diameter, settings.	May skip if snap freezing.	N/A
	Filtration (optional)	Use 0.2 µm pipette tip filter. May skip if centrifuged & stored at-80 °C.	May use if storage delay before -80 °C.	N/A
	Preservatives	Avoid if using proper centrifugation & -80 °C storage.	N/A	N/A
Sample Storage	**Short-term storage (24 hrs - 6 mos.)**	Store at 4 °C for <24 hours post-centrifuge, or -80 °C if > 24hrs.	Store at -20 °C up to 6 mos. Post-centrifugation.	
	Long-term storage (>6 mos.)	Snap-Freezing via liquid Nitrogen (no centrifugation)	Store at -80 °C (post gentle centrifugation).	Store at -20 °C or -80 °C up to 6 months.
	Thawing	Thaw aliquots at 4°C or on ice over 1-2 hours.	N/A	N/A
Sample Prep, Data Acquisition, & Reanalysis	**Buffer use**	Ensure compatibility with target metabolites; document protocol & concentration. Prepare samples day of analysis.	N/A	N/A
	Minimize Handling Time	Limit time between prep & analysis. Consider robotic prep for large studies.	N/A	N/A
	Data Acquisition	Keep samples at 4 °C during acquisition.	Document storage conditions & delays.	N/A
	Reanalysis stability	Prepare multiple aliquots post sample collection to avoid storing buffered samples or freeze-thaw cycles.	Store buffered samples at 4° C and re-run ideally within 24–48 hours, or up to 14 days.	If storage >14 days store prepared samples at -20 °C.
	Thaw inspection	Inspect on ice for precipitates. Vortex to re-dissolve.	Centrifuge & use supernatant if precipitate persists.	N/A

CHAPTER 7

DISCUSSION & CONCLUSIONS

While metabolomics has been integrated into human medicine for nearly two decades, its application to veterinary medicine has been limited by methodological inconsistencies and lack of standardized protocols. Dogs share over 360 diseases with humans and possess similar metabolic traits, environmental exposures, and dietary patterns, making them valuable comparative models. Their shorter lifespans combined with controlled environments provide unique opportunities for longitudinal research examining diet, aging, and disease development. Human urine metabolomics has well-established standards for sample collection, handling, storage, and preparation, all important factors for ensuring reproducibility and reliability in research studies. Previous studies have demonstrated that delays in storage, temperature fluctuations, and microbial contamination can significantly impact metabolite stability contributing to unreliable data and subsequent analytical challenges. In contrast, veterinary metabolomics lacks comparable standardization, with existing studies exhibiting notable methodological variation that potentially compromises reliability and cross-study comparability.

The role of advanced glycation end products (AGEs), formed during food processing and endogenously during metabolism. These compounds have been implicated in chronic disease development through protein cross-linking and receptor-mediated inflammatory pathways. Research indicates that dogs consuming commercial pet foods may ingest up to 122-fold higher AGEs per kilogram of metabolic body weight than humans on Western diets, creating a

considerable but understudied exposure that may contribute to chronic disease burden in companion animals.

Our work examined methodological considerations for canine urine metabolomics studies, with focus on sample handling, storage stability, and potential applications to nutritional research involving AGEs. We addressed gaps regarding standardization of sample collection and processing, stability assessment of AGEs, and development of a framework for pet food classification based on processing intensity and additional factors. Through systematic evaluation of existing literature, original research, and integration of human metabolomics standards, this dissertation provides foundational tools for advancing comparative research in metabolomics, nutrition, and chronic disease.

The systematic review of dog urine metabolomics studies confirmed methodological inconsistencies across published research. Analysis of 25 peer-reviewed studies published between 2007 and 2024 revealed considerable variability in sample collection, handling, storage, and preparation protocols. Of those, 12% of studies clearly defined urine collection methods and container specifications, while 56% used mixed collection methods or failed to report methodology entirely. Sample handling procedures were insufficiently detailed in 84% of studies, with one-third providing no information whatsoever. Most studies (84%) reported storage temperature but seldom documented time between collection and storage. None of the reviewed studies provided sufficient methodological detail to permit exact replication, highlighting a barrier to progress in the field. These findings highlighted need for standardized protocols that align with established human metabolomics practices while accounting for veterinary-specific considerations.

We also investigated stability of two AGEs, Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL), in canine urine under ambient storage conditions. This study evaluated whether these compounds remained stable in samples stored at room temperature (20°C) for up to 168 hours prior to freezing at -80°C. Midstream free-catch urine samples from eight healthy dogs were analyzed in duplicate using LC-MS with isotope-labeled standards. Results demonstrated that CML and CEL concentrations, as well as the CML/CEL ratio, remained stable throughout the seven-day ambient storage period. No significant changes were observed, and regression analysis showed no association between storage duration and analyte concentration. These findings indicate that these specific AGEs are robust to delayed freezing, supporting the feasibility of field-based sample collection, retrospective analysis, and biobanking initiatives where immediate ultra-low temperature storage may not be possible.

Our work also evaluated broader metabolite stability in canine urine under two common storage conditions: refrigeration (4°C) and room temperature (20°C). Using untargeted one-dimensional proton nuclear magnetic resonance (1D ^{1}H NMR) spectroscopy, 320 samples from sixteen clinically healthy dogs were analyzed over a 72-hour period. The initial dataset was reduced to six dogs due to alignment challenges, subsequent analysis of 107 consistently aligned features provided storage-dependent metabolite stability pattern information translatable to real world investigations. We found that samples stored at 4°C exhibited greater metabolite stability than those stored at 20°C, particularly beyond 24 hours. At 4°C, most features (>76%) remained within a 30% change threshold throughout the study period, while at 20°C, stability declined markedly after 48 hours, with only 58.9% of features remaining stable at 72 hours. Notably, inter-individual metabolic variability among dogs exceeded effects due to storage. The study also detailed limitations in long-term storage of buffered samples, as attempts to acquire 2D spectra

were unsuccessful. Challenges were due to precipitation within the sample after storage at -20°C after one year

We combined our findings from this work with established human metabolomics practices to develop recommendations for handling dog urine samples in metabolomics studies. These recommendations provide considerations for study design, sample collection, handling, storage, and preparation considerations specific to veterinary applications. These include standardizing collection methods, implementing consistent handling procedures to minimize cellular and bacterial degradation, and optimizing storage conditions based on intended analytical platforms. For short-term storage, refrigeration at 4°C is acceptable for up to 24-72 hours, while -20°C is suitable for up to 6 months, and -80°C is preferred for long-term storage. These guidelines provide a practical foundation for standardizing pre-analytical procedures in canine urine metabolomics and supporting more consistent, reliable research outcomes.

The integrated findings from this dissertation demonstrate need for methodological standardization in veterinary metabolomics, particularly for canine urine analysis. The systematic review demonstrated widespread inconsistency in sample handling practices, while the stability studies provided critical data to inform evidence-based protocols. We offer more complete recommendations for collection, handling, and storage of canine urine samples for metabolomics research that balance scientific rigor with practical considerations for veterinary clinical and field settings.

The observed stability of CML and CEL in canine urine stored at room temperature supports feasibility of field-based sample collection, retrospective analysis of appropriately stored biobank samples, and multi-site collaborative studies where immediate ultra-low temperature storage is not possible. Further, differential stability patterns between 4°C and 20°C

provide guidance for short-term sample handling. Therefore, stability patterns appears to be metabolite-specific, as broader untargeted analysis showed that many other metabolites exhibit variable degradation patterns under the same conditions. These also suggest that specific protocols may be necessary based on the specific metabolites or metabolite classes of interest in a given study. For general metabolomics studies, refrigeration offers superior preservation of metabolite profiles compared to ambient storage, particularly beyond 24 hours. However, extended stability of AGEs like CML and CEL even at room temperature suggests that these compounds may be valuable biomarkers when optimal storage conditions are unavailable. This work enhances utility of AGE measurements in clinical veterinary practice, field studies, and biobanking initiatives. By improving the reliability and reproducibility of canine urine metabolomics, these tools enhance the value of dogs as comparative models for studying chronic disease development. The extended stability of urinary AGEs further supports their potential utility as biomarkers of dietary exposure and metabolic health in both veterinary and human contexts.

There are limitations to this research. The systematic review was constrained by the relatively small number of published canine urine metabolomics studies, reflecting the nascent state of the field. Future reviews should assess methodologies for of bio samples. Our NMR-based pilot study faced significant challenges with spectral alignment and feature matching across individuals, limiting the final dataset to six dogs and reducing statistical power. Additionally, the loss of 2D NMR capability for stored samples highlights storage limitations that warrant further investigation. The AGE stability study focused specifically on CML and CEL, two widely studied but not comprehensive representatives of the entire AGE class. Future work should conduct stability assessments to include additional AGEs, as well as their

precursors, to develop a more complete understanding of the pre-analytical behavior of AGEs in urine and other biofluids. Similarly, the storage conditions evaluated (4°C, 20°C, -80°C) represent common research scenarios but do not address more variable field conditions such as temperature fluctuations, freeze-thaw cycles, or shipping effects. Future studies considering such real-world variables would enhance applicability of findings.

Future research directions should focus on several key areas to build upon this foundational work. First, validation of the proposed sample handling and storage recommendations across diverse dog populations, including different breeds, ages, and disease states, would strengthen their generalizability. Second, comprehensive profiling of urinary AGEs in dogs consuming different diet types would establish reference ranges and clarify relationships between dietary exposure, endogenous formation, and excretion patterns. Third, longitudinal studies correlating urinary AGE profiles with health outcomes would clarify their utility as biomarkers of chronic disease risk in dogs.

This work addressed methodological gaps in canine urine metabolomics, focusing on pre-analytical variables influencing sample integrity and biomarker stability. We provide foundational tools to improve reproducibility and reliability in veterinary metabolomics. The demonstrated stability of urinary AGEs under various storage conditions supports their potential utility as biomarkers of dietary exposure and metabolic health. By improving our ability to reliably measure metabolic signatures in dogs, this work highlights the value of dogs as translational models for studying relationships between diet and health outcomes.

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APPENDICES

Appendix A (pt 1 of 4)

Year Published	Title	Authors	doi	Metabolomics Platform	Was the sample collection procedure clearly defined?	Was the sample collection container manufacturer & type provided?	Was the sample collection method the same for each subject?	Was the sample storage preparation clearly defined?	Was the aliquot amount, sample storage container/type & manufacturer provided?	Was the sample storage condition clearly defined?	Sample Storage Condition Temperature	Was the time between collection and storage clearly defined?	Was the sample thawing method clearly defined?	Was the sample preparation for data acquisition clearly defined for repeatability?	Was the amount of reagent(s) manufactuerer/ source provided?	Was the manufacterer/source for sample preparation containers and data acusition tubes provided?	Were the samples temperature controlled prior to data capture?	Were samples temperature controlled during data acquisition?
2007	Validation of a urine metabolome fingerprint in dog for phenotypic classification	Viant, et., al	https://doi.org/10.1007/s11306-007-	NMR	Yes	Yes	Yes	No	No	Yes	-80°C	Yes	No	No	No	No	Unknown	Unknown
2007	Metabonomic investigations of aging and caloric restriction in a life-long dog study	Wang, et., al	10.1021/p	NMR	No	No	No	No	No	Yes	-20C	No	No	No	No	No	Unknown	Unknown
2010	Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs	Beckmann et., al	10.1017/S000711450999300X	MS	Yes	Yes	Yes	No	No	Yes	-80°C	Yes	No	No	No	No	Yes	Yes
2012	NMR-based metabolomics study of canine bladder cancer	Zhang, et., al	10.1016/j.bbadis.2012.08.001	NMR	No	No	Yes	No	No	Yes	-80°C	No	No	Yes	No	No	Unknown	Unknown
2015	Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea	Guard, et., al.	10.1371/journal.pone.0127259	UPLC-MS and HPLC-MS	Yes	No	Yes	No	No	Yes	-80°C	No	No	No	No	No	Unknown	Yes
2015	Consumption of Cooked Navy Bean Powders Modulate the Canine Fecal and Urine Metabolome	Forster, et., al.	10.2174/2213235X03666615051923 4354	LC-MS	No	No	No	No	No	Yes	-80°C	No	No	No	No	No	Unknown	Unknown
2017	The urine metabolome differs between lean and overweight Labrador Retriever dogs during a feed-challenge	Söder, et., al.	10.1371/journal.pone.0180086	NMR	Yes	Yes	Yes	Yes	Yes	Yes	-70°C	Yes	No	No	No	No	Unknown	Unknown
2018	Species differences in bile acids I. Plasma and urine bile acid composition	Thakare, et., al	10.1002/jat.3644	LC-MS/MS	No	No	Unknown	No	No	No	Unknown	No	No	Yes	Yes	No	Unknown	Unknown

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Year Published	Title	Authors	doi	Metabolomics Platform	Was the sample collection procedure clearly defined?	Was the sample collection container manufacturer & type provided?	Was the sample collection method the same for each subject?	Was the sample storage preparation clearly defined?	Was the aliquot amount, sample storage container/type & manufacturer provided?	Was the sample storage condition clearly defined?	Sample Storage Condition Temperature	Was the time between collection and storage clearly defined?	Was the sample thawing method clearly defined?	Was the sample preparation for data acquisition clearly defined for repeatability?	Was the amount of reagent(s) manufactuerer/ source provided?	Was the manufacterer/source for sample preparation containers and data acusition tubes provided?	Were the samples temperature controlled prior to data capture?	Were samples temperature controlled during data acquisition?
2019	Untargeted metabolomic profiling of urine from healthy dogs and dogs with chronic hepatic disease	Lawrence, et., al.	10.1371/journal.pone.0217797	GC-MS	Yes	No	Yes	No	Yes	-80°C	Yes	No	No	No	No	Unknown	Unknown	Unknown
2020	Specific urinary metabolites in canine mammary gland tumors	Valko-Rokytovská, et., al.	10.4142/jvs.2020.21.e23	UHPLC	No	No	No	No	Yes	-50°C	No	No	No	Yes	No	Unknown	Unknown	Unknown
2020	Targeted Metabolomics With Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS) Highlights Metabolic Differences in Healthy and Atopic Staffordshire Bull Terriers Fed Two Different Diets, A Pilot Study	Moore, et., al	10.3389/fvets.2020.554296	UPLC-MS/MS	Yes	No	Unknown	Yes	No	-80°C	No	No	No	No	No	Unknown	Unknown	Unknown
2020	Urinary proteome and metabolome in dogs (Canis lupus familiaris): The effect of chronic kidney disease	Ferlizza, et., al	10.1016/j.jprot.2020.103795	NMR	Yes	No	Yes	No	No	-80°C	No	No	No	No	No	Unknown	Unknown	Unknown
2020	Varying Protein Levels Influence Metabolomics and the Gut Microbiome in Healthy Adult Dogs	Ephraim, et., al	10.3390/toxins12080517	UHPLC-MS/MS	No	No	Unknown	No	No	Unknown	No	No	No	No	No	Unknown	Unknown	Unknown
2021	Combined Untargeted and Targeted Metabolomics Approaches Reveal Urinary Changes of Amino Acids and Energy Metabolism in Canine Babesiosis With Different Levels of Kidney Function	Kuleš, et., al	10.3389/fmicb.2021.715701	LC-MS/MS and FIA-MS	Yes	No	Yes	No	No	Yes	-80°C	No	No	No	Yes	No	Unknown	Unknown

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Year Published	Title	Authors	doi	Metabolomics Platform	Was the sample collection procedure clearly defined?	Was the sample collection container manufacturer & type provided?	Was the sample collection method the same for each subject?	Was the sample storage preparation clearly defined?	Was the aliquot amount, sample storage container/type & manufacturer provided?	Was the sample storage condition clearly defined?	Sample Storage Condition Temperature	Was the time between collection and storage clearly defined?	Was the sample thawing method clearly defined?	Was the sample preparation for data acquisition clearly defined for repeatability?	Was the amount of reagent(s) manufacturer/ source provided?	Was the manufacterer/source for sample preparation containers and data acusition tubes provided?	Were the samples temperature controlled prior to data capture?	Were samples temperature controlled during data acquisition?
2021	Evaluation of Serum and Urine Amino Acids in Dogs with Chronic Kidney Disease and Healthy Dogs Fed a Renal Diet	Brunetto, et., al	10.3390/metabo1120844	HPLC	No	No	No	No	Yes	-80°C	No	No	No	No	No	Unknown	Unknown	Unknown
2022	(1)H NMR based urinary metabolites profiling dataset of canine mammary tumors	Lee, et., al	10.1038/s41597-022-01229-1	NMR	No	No	No	No	Yes	-80°C	No	No	No	Yes	No	Yes	Yes	Yes
2022	Profound Perturbation in the Metabolome of a Canine Obesity and Metabolic Disorder Model	Qu, et., al	10.3389/fendo.2022.849060	UHPLC-MS	No	No	Unknown	No	No	Unknown	No	No	No	No	No	Yes	Yes	Yes
2022	Untargeted Metabolomics Identify a Panel of Urinary Biomarkers for the Diagnosis of Urothelial Carcinoma of the Bladder, as Compared to Urolithiasis with or without Urinary Tract Infection in Dogs	Tsamouri, et., al	10.3390/metabo12030200	MS	No	No	Unknown	Yes	No	Yes	-80°C	No	No	No	No	Unknown	Unknown	Unknown
2022	Urinary cortisol metabolites are reduced in MDR1 mutant dogs in a pilot targeted GC-MS urinary steroid hormone metabolome analysis	Gramer, et., al	10.1111/jvp.13050	GC-MS	No	No	Yes	No	No	Unknown	No	No	No	Yes	No	Unknown	Unknown	Unknown
2022	Comprehensive profiling of lipid metabolites in urine of canine patients with liver mass	Kida, et., al	10.1292/jvms.21-0373	LC-MS/MS	No	No	Unknown	No	Yes	-28°C	No	No	No	No	No	Unknown	Unknown	Unknown
2022	The profile of urinary lipid metabolites in healthy dogs	Kida, et., al	10.1292/jvms.22-0373	LC-MS/MS	No	No	Unknown	No	No	-28°C	No	No	No	No	No	Unknown	Unknown	Unknown
2023	Comparative plasma and urine metabolomics analysis of juvenile and adult canines	Wu, et., al	10.3389/fvets.2022.1037327	UHPLC-Q-TOFMS	Yes	No	Yes	No	No	-80°C	No	No	No	No	No	Unknown	Unknown	Unknown

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Year Published	Title	Authors	doi	Metabolomics Platform	Was the sample collection procedure clearly defined?	Was the sample collection container manufacturer & type provided?	Was the sample collection method the same for each subject?	Was the sample storage preparation clearly defined?	Was the aliquot amount, sample storage container/type & manufacturer provided?	Was the sample storage condition clearly defined?	Sample Storage Condition Temperature	Was the time between collection and storage clearly defined?	Was the sample thawing method clearly defined?	Was the sample preparation for data acquisition clearly defined for repeatability?	Was the amount of reagent(s) manufactuerer/ source provided?	Was the manufacterer/source for sample preparation containers and data acusition tubes provided?	Were the samples temperature controlled prior to data capture?	Were samples temperature controlled during data acquisition?
2023	Untargeted metabolomic profiles reveal widespread metabolic perturbations and identify candidate biomarkers in aminoaciduric canine hypoaminoacidemic hepatopathy syndrome	Loftus, et., al.	10.2460/ajvr.23.08.0186	LC-MS/MS	No	No	No	No	No	Yes	-80°C	No	No	No	No	No	Unknown	Unknown
2024	A longitudinal study of the blood and urine metabolome of Vipera berus envenomated dogs	Nicolaysen, et., al	10.1016/j.rvsc.2024.105287	LC-MS/MS	No	No	Yes	No	No	Yes	-80°C	Yes	No	No	No	No	Yes	Uknown
2024	Candidate urinary biomarkers show promise for distinguishing between calcium oxalate versus struvite urolithiasis in dogs	Xu, et., al	10.2460/ajvr.23.09.0214	UPLC-MSMS	No	No	No	No	No	Yes	-80°C	No	No	No	No	No	Unknown	Unknown

Appendix B

Dog ID	Time (hr)	Rep	CML mM	CEL mM	CML ug/mL	CEL ug/mL	CML/CEL ug/mL
1	0	1	0.0188	0.0071	3.846	1.545	2.490
1	0	2	0.0191	0.0069	3.923	1.513	2.593
1	1	1	0.0266	0.0084	5.448	1.828	2.981
1	1	2	0.0247	0.0083	5.055	1.803	2.803
1	3	1	0.0184	0.0055	3.766	1.200	3.139
1	3	2	0.0217	0.0077	4.447	1.669	2.665
1	6	1	0.0352	0.0122	7.209	2.651	2.720
1	6	2	0.0227	0.0082	4.652	1.784	2.608
1	12	1	0.0384	0.0116	7.871	2.533	3.108
1	12	2	0.0234	0.0086	4.805	1.882	2.553
1	24	1	0.0221	0.0081	4.533	1.764	2.570
1	24	2	0.0209	0.0073	4.294	1.595	2.692
1	36	1	0.0236	0.0077	4.846	1.675	2.892
1	36	2	0.0219	0.0083	4.498	1.806	2.491
1	48	1	0.0228	0.0073	4.666	1.586	2.942
1	48	2	0.0234	0.0075	4.789	1.626	2.945
1	72	1	0.0263	0.0099	5.386	2.167	2.485
1	72	2	0.0233	0.0078	4.771	1.711	2.788
1	120	1	0.0223	0.0081	4.576	1.774	2.580
1	120	2	0.0228	0.0079	4.682	1.719	2.724

1	168	1	0.0242	0.0080	4.968	1.735	2.863
1	168	2	0.0238	0.0078	4.875	1.711	2.849
2	0	1	0.0194	0.0048	3.970	1.038	3.827
2	0	2	0.0187	0.0046	3.826	1.006	3.805
2	1	1	0.0199	0.0047	4.075	1.032	3.948
2	1	2	0.0189	0.0051	3.882	1.108	3.502
2	3	1	0.0180	0.0043	3.696	0.943	3.919
2	3	2	0.0171	0.0048	3.507	1.040	3.371
2	6	1	0.0429	0.0131	8.788	2.859	3.074
2	6	2	0.0207	0.0055	4.237	1.201	3.528
2	12	1	0.0169	0.0037	3.472	0.808	4.299
2	12	2	0.0166	0.0044	3.406	0.953	3.573
2	24	1	0.0180	0.0045	3.693	0.983	3.758
2	24	2	0.0192	0.0050	3.928	1.085	3.620
2	36	1	0.0223	0.0059	4.581	1.280	3.579
2	36	2	0.0204	0.0048	4.188	1.057	3.962
2	48	1	0.0205	0.0046	4.194	0.995	4.217
2	48	2	0.0177	0.0052	3.620	1.136	3.187
2	72	1	0.0226	0.0051	4.639	1.120	4.141
2	72	2	0.0214	0.0052	4.391	1.124	3.905
2	120	1	0.0191	0.0046	3.915	1.009	3.880
2	120	2	0.0181	0.0044	3.704	0.964	3.844
2	168	1	0.0167	0.0041	3.431	0.902	3.803

2	168	2	0.0179	0.0041	3.665	0.890	4.120
3	0	1	0.0204	0.0049	4.180	1.070	3.905
3	0	2	0.0182	0.0055	3.740	1.202	3.112
3	1	1	0.0187	0.0051	3.842	1.110	3.462
3	1	2	0.0175	0.0049	3.596	1.070	3.361
3	3	1	0.0180	0.0049	3.683	1.057	3.483
3	3	2	0.0186	0.0050	3.806	1.084	3.512
3	6	1	0.0194	0.0053	3.980	1.158	3.437
3	6	2	0.0170	0.0045	3.489	0.974	3.582
3	12	1	0.0238	0.0059	4.873	1.280	3.806
3	12	2	0.0174	0.0054	3.567	1.181	3.021
3	24	1	0.0179	0.0040	3.670	0.866	4.239
3	24	2	0.0175	0.0049	3.592	1.070	3.358
3	36	1	0.0141	0.0038	2.895	0.820	3.533
3	36	2	0.0149	0.0045	3.063	0.988	3.102
3	48	1	0.0175	0.0048	3.588	1.051	3.413
3	48	2	0.0167	0.0045	3.421	0.973	3.517
3	72	1	0.1071	0.0271	21.948	5.908	3.715
3	72	2	0.0239	0.0059	4.898	1.279	3.829
3	120	1	0.0152	0.0042	3.117	0.906	3.442
3	120	2	0.0122	0.0029	2.503	0.633	3.951
3	168	1	0.0349	0.0083	7.147	1.816	3.936
3	168	2	0.0122	0.0029	2.503	0.633	3.951

4	0	1	0.1305	0.0481	26.762	10.484	2.553
4	0	2	0.0347	0.0125	7.110	2.730	2.604
4	1	1	0.0439	0.0097	8.997	2.107	4.271
4	1	2	0.0327	0.0126	6.709	2.758	2.433
4	3	1	0.0461	0.0251	9.441	5.473	1.725
4	3	2	0.0329	0.0123	6.746	2.672	2.525
4	6	1	0.0307	0.0110	6.301	2.407	2.618
4	6	2	0.0322	0.0122	6.606	2.657	2.486
4	12	1	0.0332	0.0108	6.810	2.348	2.900
4	12	2	0.0336	0.0126	6.896	2.752	2.506
4	24	1	0.0343	0.0124	7.028	2.696	2.607
4	24	2	0.0336	0.0126	6.896	2.752	2.506
4	36	1	0.0297	0.0105	6.089	2.289	2.660
4	36	2	0.0302	0.0101	6.192	2.208	2.804
4	48	1	0.0401	0.0168	8.227	3.653	2.252
4	48	2	0.0314	0.0125	6.436	2.733	2.355
4	72	1	0.0261	0.0096	5.347	2.082	2.568
4	72	2	0.0263	0.0094	5.382	2.060	2.613
4	120	1	0.0318	0.0108	6.525	2.362	2.763
4	120	2	0.0293	0.0102	6.006	2.216	2.710
4	168	1	0.0380	0.0144	7.797	3.137	2.485
4	168	2	0.0380	0.0126	7.797	2.755	2.830
5	0	1	0.2258	0.0371	46.279	8.097	5.715

5	0	2	0.2066	0.0314	42.358	6.852	6.182
5	1	1	0.1975	0.0322	40.495	7.027	5.763
5	1	2	0.1779	0.0299	36.478	6.524	5.591
5	3	1	0.3684	0.0569	75.528	12.406	6.088
5	3	2	0.2064	0.0317	42.312	6.909	6.124
5	6	1	0.2187	0.0329	44.830	7.178	6.245
5	6	2	0.2003	0.0331	41.071	7.207	5.699
5	12	1	0.3932	0.0621	80.608	13.539	5.954
5	12	2	0.2167	0.0328	44.413	7.144	6.217
5	24	1	0.2169	0.0329	44.470	7.182	6.192
5	24	2	0.2006	0.0341	41.128	7.427	5.538
5	36	1	0.4732	0.0692	97.004	15.094	6.427
5	36	2	0.2278	0.0339	46.704	7.391	6.319
5	48	1	0.4820	0.0783	98.810	17.065	5.790
5	48	2	0.2209	0.0350	45.294	7.631	5.936
5	72	1	0.3376	0.0508	69.198	11.067	6.253
5	72	2	0.2343	0.0344	48.036	7.503	6.402
5	120	1	0.2072	0.0333	42.482	7.265	5.847
5	120	2	0.2063	0.0322	42.292	7.012	6.032
5	168	1	0.2085	0.0312	42.747	6.796	6.290
5	168	2	0.2158	0.0316	44.238	6.880	6.430
6	0	1	0.2578	0.0377	52.844	8.228	6.422
6	0	2	0.2577	0.0379	52.823	8.268	6.389

6	1	1	0.2718	0.0406	55.721	8.849	6.297
6	1	2	0.2679	0.0417	54.928	9.084	6.047
6	3	1	0.2875	0.0473	58.930	10.303	5.720
6	3	2	0.2804	0.0425	57.490	9.268	6.203
6	6	1	0.3191	0.0479	65.410	10.448	6.261
6	6	2	0.2772	0.0423	56.828	9.213	6.168
6	12	1	0.3725	0.0568	76.368	12.391	6.163
6	12	2	0.3255	0.0493	66.738	10.755	6.205
6	24	1	0.1854	0.0277	38.002	6.045	6.286
6	24	2	0.1687	0.0232	34.589	5.059	6.837
6	36	1	0.1764	0.0270	36.171	5.883	6.148
6	36	2	0.1778	0.0255	36.458	5.548	6.571
6	48	1	0.1835	0.0246	37.618	5.371	7.004
6	48	2	0.1838	0.0259	37.673	5.652	6.666
6	72	1	0.1880	0.0263	38.538	5.728	6.728
6	72	2	0.1920	0.0276	39.362	6.018	6.541
6	120	1	0.2459	0.0356	50.408	7.760	6.496
6	120	2	0.1917	0.0275	39.297	5.987	6.563
6	168	1	0.1666	0.0221	34.153	4.813	7.096
6	168	2	0.1679	0.0229	34.415	4.998	6.885
7	0	1	0.0646	0.0082	13.251	1.793	7.390
7	0	2	0.0491	0.0068	10.065	1.475	6.825
7	1	1	0.0452	0.0058	9.270	1.261	7.349

7	1	2	0.0472	0.0059	9.673	1.295	7.469
7	3	1	0.0506	0.0066	10.383	1.436	7.230
7	3	2	0.0464	0.0060	9.520	1.301	7.318
7	6	1	0.0273	0.0037	5.605	0.802	6.992
7	6	2	0.0266	0.0034	5.453	0.745	7.322
7	12	1	0.0557	0.0074	11.425	1.620	7.051
7	12	2	0.0415	0.0058	8.513	1.265	6.732
7	24	1	0.0542	0.0069	11.105	1.502	7.395
7	24	2	0.0452	0.0061	9.267	1.338	6.926
7	36	1	0.0572	0.0077	11.734	1.680	6.983
7	36	2	0.0442	0.0058	9.063	1.266	7.156
7	48	1	0.0889	0.0122	18.229	2.667	6.834
7	48	2	0.0512	0.0062	10.498	1.353	7.761
7	72	1	0.0418	0.0053	8.566	1.151	7.445
7	72	2	0.0432	0.0054	8.856	1.167	7.588
7	120	1	0.0700	0.0086	14.353	1.879	7.638
7	120	2	0.0491	0.0063	10.075	1.377	7.315
7	168	1	0.0815	0.0100	16.711	2.177	7.676
7	168	2	0.0499	0.0066	10.220	1.439	7.100
8	0	1	0.0983	0.0274	20.146	5.984	3.367
8	0	2	0.0869	0.0246	17.813	5.371	3.316
8	1	1	0.0575	0.0178	11.784	3.884	3.034
8	1	2	0.0609	0.0165	12.486	3.604	3.465

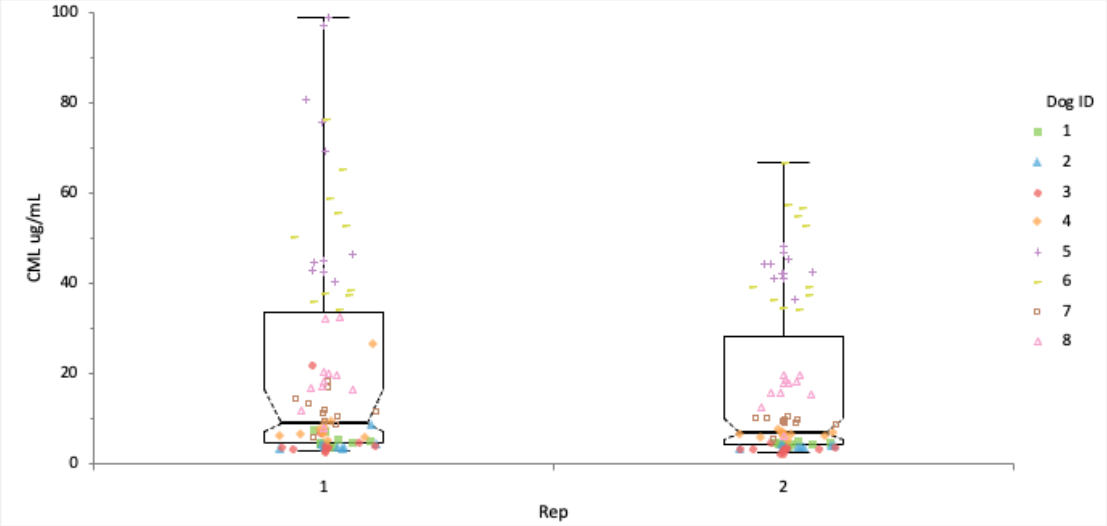
8	3	1	0.0805	0.0220	16.510	4.792	3.445
8	3	2	0.0745	0.0215	15.277	4.692	3.256
8	6	1	0.0818	0.0219	16.765	4.782	3.506
8	6	2	0.0775	0.0221	15.878	4.819	3.295
8	12	1	0.0989	0.0269	20.267	5.867	3.455
8	12	2	0.0960	0.0244	19.677	5.314	3.703
8	24	1	0.0379	0.0106	7.771	2.316	3.356
8	24	2	0.0295	0.0084	6.049	1.834	3.298
8	36	1	0.0839	0.0234	17.191	5.093	3.375
8	36	2	0.0771	0.0225	15.802	4.897	3.227
8	48	1	0.1563	0.0484	32.047	10.562	3.034
8	48	2	0.0900	0.0260	18.459	5.674	3.253
8	72	1	0.0893	0.0244	18.308	5.310	3.448
8	72	2	0.0871	0.0240	17.864	5.243	3.407
8	120	1	0.1585	0.0508	32.486	11.075	2.933
8	120	2	0.0961	0.0256	19.704	5.573	3.536
8	168	1	0.0959	0.0262	19.656	5.718	3.437
8	168	2	0.0882	0.0249	18.076	5.419	3.336

Appendix B

CML ug/mL by Replicate

Comparison of replicate 1 versus replicate 2 - all time points and all dogs - overall statistically no significant difference

Descriptives



N	176

CML ug/mL by Rep	Minimum	1st Quartile	Median	95% CI	3rd Quartile	Maximum
1	2.895	4.6500	9.1338	7.0281 to 16.5102	33.4582	98.810
2	2.503	4.3342	7.0032	5.4526 to 10.0749	28.2853	66.738

Location

Kruskal-Wallis test

CML ug/mL by Rep	N	Rank sum	Mean rank
1	88	8204.0	93.23
2	88	7372.0	83.77

H statistic	1.52
X^2 approximation	1.52
DF	1
p-value	0.2184

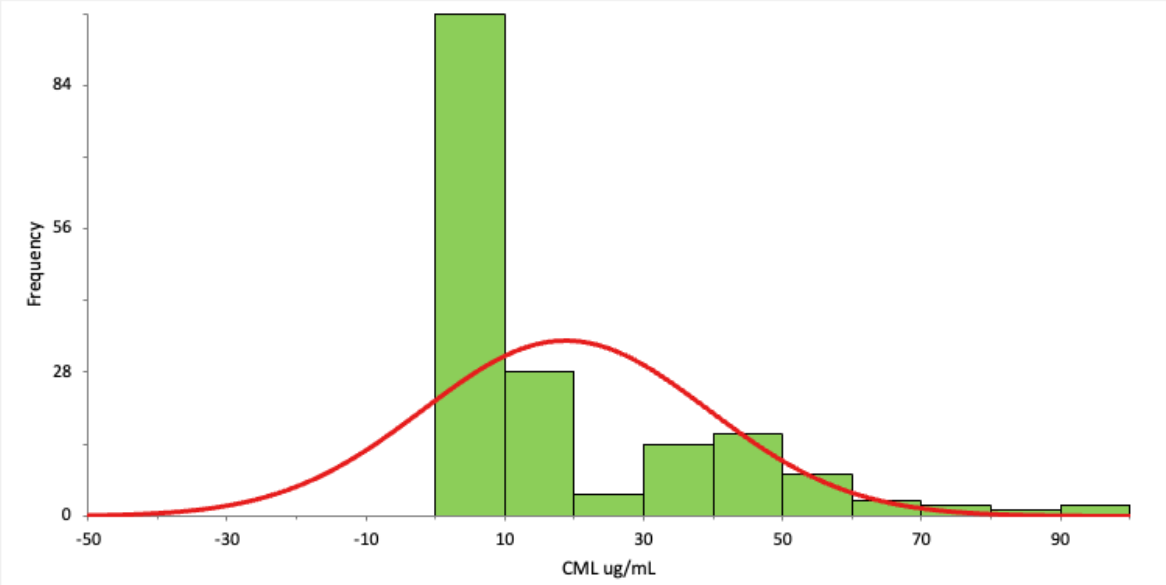
H0: $\theta_1 = \theta_2 = \theta...$
The median of the populations are all equal.
H1: $\theta_i \neq \theta_j$ for at least one i,j
The median of the populations are not all equal.
[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Distribution: CML ug/mL

histogram of CML data all dogs at all time points with both replicates

Descriptives

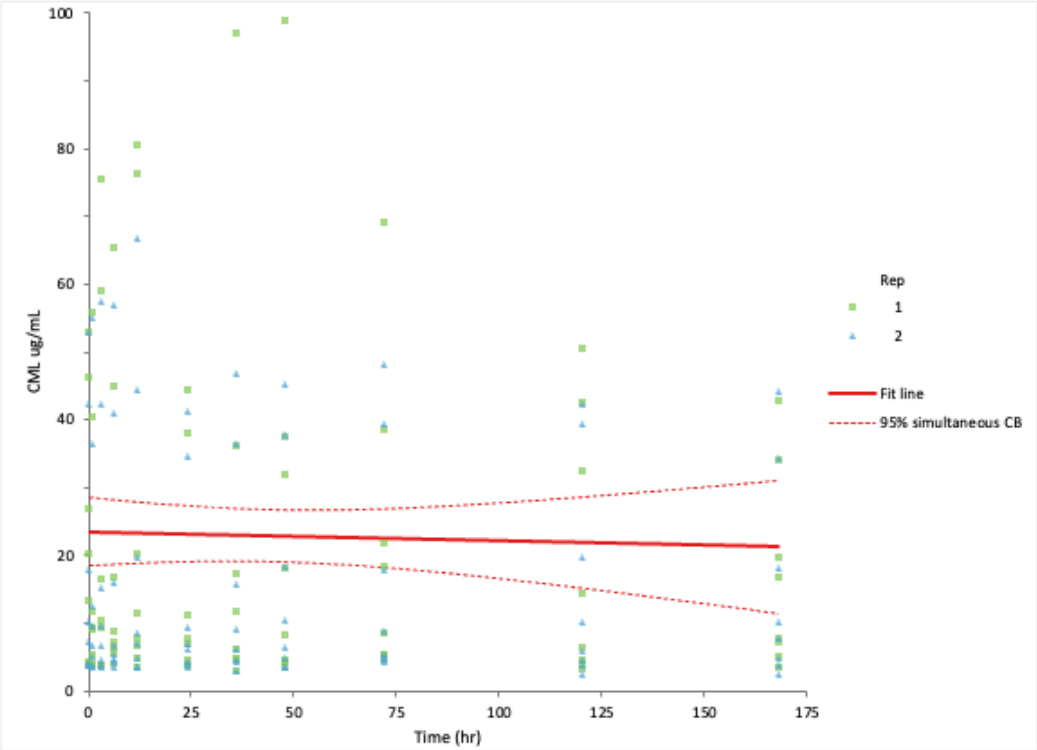


Appendix B

Fit: CML ug/mL

CML - linear model fit - all dogs at all time points with both replicates (showing replicates as differentcolors) - can change this to show individual dogs

No time effect, ressiduals are not significantly different.



N	176
Mean of Y	22.8998

Variable	Mean	SD	Minimum	Median	Maximum
Time (hr)	44.5	52.7	0	24.0	168

Fit

Equation	CML ug/mL = 23.51 - 0.01379 Time (hr)
Weight	Dog ID

R^2	0.001
R^2 adjusted	-0.005
RMSE	44.11081

Parameter	Estimate	95% CI	SE	t	p-value
Constant	23.51	19.46 to 27.57	2.0552	11.44	<0.0001[1]
Time	-0.01379	-0.07268 to 0.04511	0.029842	-0.46	0.6447[2]

H0: β = 0
The parameter is equal to 0.
H1: β ≠ 0
The parameter is not equal to 0.
[1] Reject the null hypothesis in favour of the alternative hypothesis at the 5% significance level.
[2] Do not reject the null hypothesis at the 5% significance level.

Appendix B

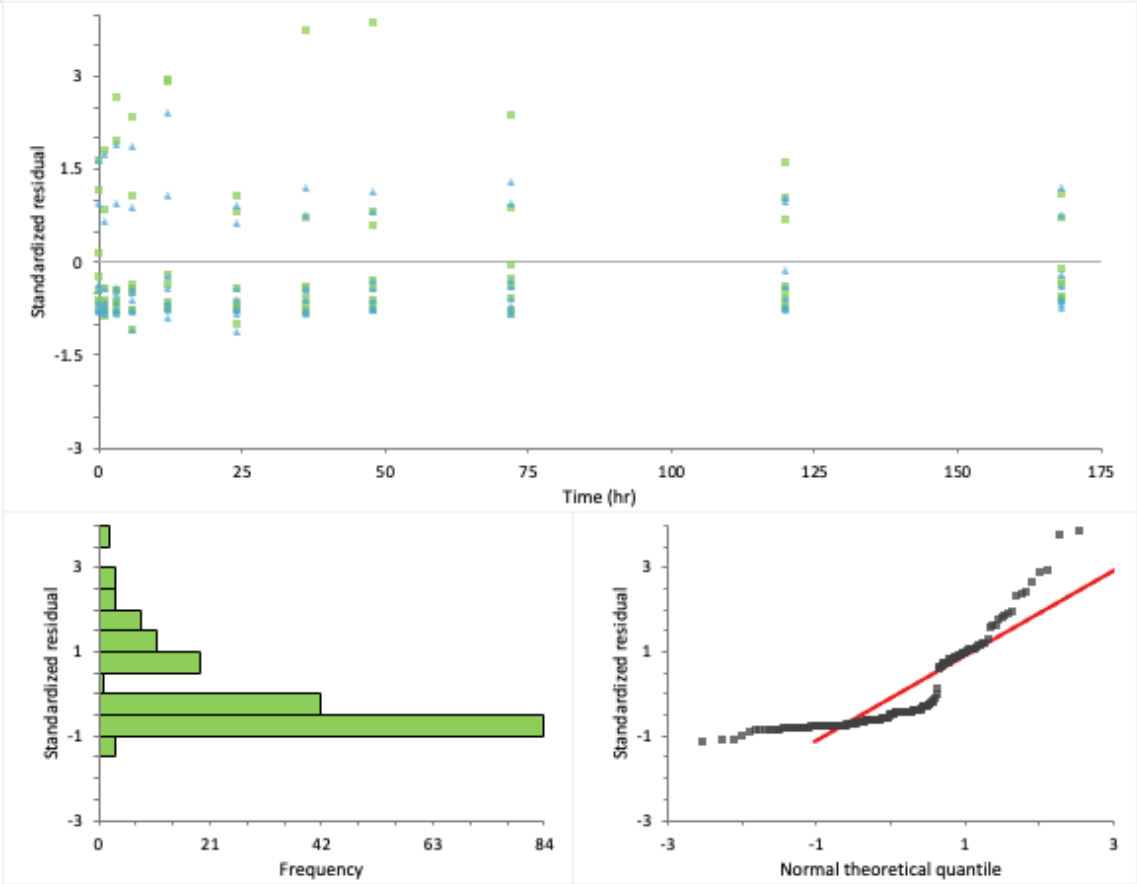
Fit: CML ug/mL (cont.)

Effect of Model

Source	SS	DF	MS	F	p-value
Difference	415.2340	1	415.2340	0.21	0.6447
Error	338562.8694	174	1945.7636		
Null model	338978.1033	175	1937.0177		

H0: E(Y|X=x) = μ
The model is no better than a null model Y=μ.
H1: E(Y|X=x) = α + βx
The model is better than the null model.

Residuals



Normality

Shapiro-Wilk test

W statistic	0.76
p-value	<0.0001[1]

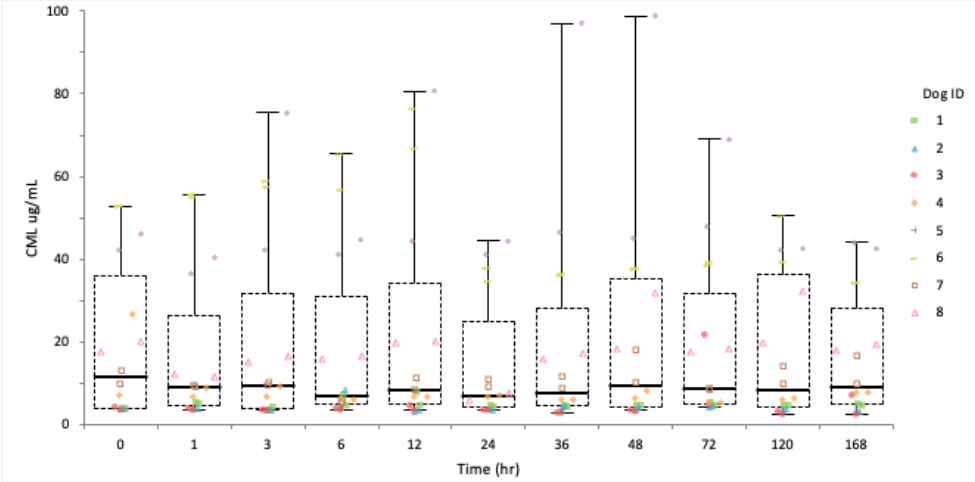
H0: F(e) = N(μ, σ)
The distribution of the population is normal with unspecified mean and standard deviation.
H1: F(e) ≠ N(μ, σ)
The distribution of the population is not normal.
[1] Reject the null hypothesis in favour of the alternative hypothesis at the 10% significance level.

Appendix B

Comparison of Groups: CML by Time

CML box plots - black line is median and dogs are colored individually. No effect on time. Pairwise comparison between time points are not significant

Descriptives



N	176

CML ug/mL by Time (hr)	Minimum	1st Quartile	Median	95% CI	3rd Quartile	Maximum
0	3.740	3.9426	11.6580	3.9228 to 42.3578	35.8594	52.844
1	3.596	4.4835	9.1338	4.0754 to 36.4776	26.4810	55.721
3	3.507	3.7826	9.4805	3.7660 to 42.3116	31.5610	75.528
6	3.489	4.9857	6.9077	4.6522 to 41.0715	30.9438	65.410
12	3.406	4.8329	8.1919	4.8045 to 44.4133	34.3525	80.608
24	3.592	4.0807	6.9621	3.9285 to 34.5889	24.8039	44.470
36	2.895	4.5327	7.6278	4.4980 to 36.1714	28.2628	97.004
48	3.421	4.3904	9.3624	4.1936 to 37.6184	35.2969	98.810
72	4.391	5.0848	8.7108	4.8976 to 38.5379	31.6255	69.198
120	2.503	4.1904	8.3000	3.9149 to 39.2970	36.4591	50.408
168	2.503	4.9133	9.0085	4.8746 to 34.1526	28.1122	44.238

Quantiles	0.05	0.95
0	3.7533	52.8404
1	3.6328	55.6017
3	3.5334	73.0386
6	3.5625	64.1226
12	3.4157	79.9723
24	3.6036	43.9691
36	2.9205	89.4588
48	3.4462	90.7824
72	4.4281	66.0240
120	2.5950	49.2191
168	2.6423	44.0141

Appendix B

Comparison of Groups: CML by Time (cont.)

Location

Kruskal-Wallis test

CML ug/mL by Time (hr)	N	Rank sum	Mean rank
0	16	1473.0	92.06
1	16	1414.0	88.38
3	16	1393.0	87.06
6	16	1441.0	90.06
12	16	1491.5	93.22
24	16	1250.5	78.16
36	16	1359.0	84.94
48	16	1423.0	88.94
72	16	1546.0	96.63
120	16	1362.5	85.16
168	16	1422.5	88.91

H statistic	1.46
X^2 approximation	1.46
DF	10
p-value	0.9991

H0: $\theta_1 = \theta_2 = \theta...$

The median of the populations are all equal.

H1: $\theta_i \neq \theta_j$ for at least one i,j

The median of the populations are not all equal.

[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Comparison of Groups: CML by Time (cont.)

Multiple Comparisons

Steel-Dwass-Critchlow-Fligner all pairs comparisons

Contrast	Hodges-Lehmann location shift	Simultaneous 95% CI	0	p-value
12 - 24	1.2077	-23.1637 to 40.7205		0.9999
72 - 24	1.1919	-20.1379 to 31.6418		0.9915
168 - 24	0.9957	-21.4726 to 27.1244		1.0000
0 - 24	0.9295	-17.8567 to 36.3092		0.9987
72 - 36	0.8666	-27.8053 to 30.8449		0.9998
0 - 36	0.8523	-26.3929 to 34.5450		1.0000
1 - 24	0.8380	-26.2180 to 30.4291		0.9999
72 - 120	0.8079	-27.7150 to 28.3317		1.0000
12 - 6	0.7430	-33.2004 to 38.9607		1.0000
12 - 36	0.6781	-27.6586 to 40.1966		1.0000
48 - 24	0.6696	-19.5436 to 31.5699		1.0000
3 - 24	0.6085	-25.0690 to 38.0179		1.0000
72 - 6	0.5963	-32.2158 to 31.2705		1.0000
36 - 24	0.5826	-25.3261 to 30.1228		1.0000
168 - 36	0.5687	-28.3743 to 25.0891		1.0000
0 - 120	0.5303	-28.5158 to 28.0044		1.0000
48 - 36	0.5253	-27.9447 to 29.4919		1.0000
0 - 168	0.4522	-16.6014 to 32.1381		1.0000
120 - 24	0.4324	-20.2355 to 32.4009		1.0000
168 - 120	0.3067	-28.8214 to 19.7992		1.0000
168 - 6	0.2688	-31.2573 to 25.9820		1.0000
1 - 36	0.2575	-29.4625 to 28.7611		1.0000
48 - 120	0.2458	-28.2924 to 30.9407		1.0000
0 - 48	0.2321	-28.2008 to 27.8204		1.0000
0 - 1	0.1875	-23.2262 to 34.4948		1.0000
0 - 3	0.1844	-33.1705 to 32.9168		1.0000
1 - 120	0.1808	-30.2996 to 23.2344		1.0000
48 - 168	0.1727	-16.1860 to 29.5437		1.0000
3 - 120	0.1613	-28.9791 to 36.2313		1.0000
3 - 36	0.1046	-27.0165 to 37.8137		1.0000
72 - 12	0.0731	-39.5158 to 27.9365		1.0000
120 - 36	0.0657	-29.6462 to 29.5907		1.0000
3 - 1	0.0623	-27.0366 to 37.2568		1.0000
0 - 12	0.0260	-39.9761 to 32.5761		1.0000
1 - 168	0.0093	-24.8823 to 26.2579		1.0000
48 - 1	0.0082	-22.8816 to 30.9095		1.0000
48 - 6	-0.0147	-27.7914 to 31.0120		1.0000
0 - 6	-0.0572	-27.8200 to 35.7513		1.0000
3 - 48	-0.1668	-28.5397 to 37.5227		1.0000
48 - 12	-0.1760	-39.7474 to 29.1056		1.0000
3 - 168	-0.1791	-24.6326 to 37.4371		1.0000
3 - 6	-0.2096	-31.6305 to 36.7071		1.0000
1 - 6	-0.2578	-33.0461 to 29.8711		1.0000
168 - 12	-0.3937	-40.9820 to 22.9896		1.0000
36 - 6	-0.4450	-33.0963 to 29.8702		1.0000
48 - 72	-0.6925	-28.8636 to 28.8174		1.0000
120 - 6	-0.7106	-30.4770 to 30.5092		1.0000
168 - 72	-0.7474	-29.1420 to 20.7992		1.0000
0 - 72	-0.7552	-25.2865 to 30.8748		1.0000
1 - 72	-0.7757	-29.6887 to 27.6219		1.0000
1 - 12	-0.8597	-40.1132 to 28.6066		1.0000
24 - 6	-0.9393	-35.0229 to 25.8012		0.9999
3 - 72	-0.9605	-29.0179 to 36.9296		1.0000
120 - 12	-0.9633	-39.7313 to 29.0802		1.0000
3 - 12	-1.0186	-39.9659 to 35.5018		1.0000

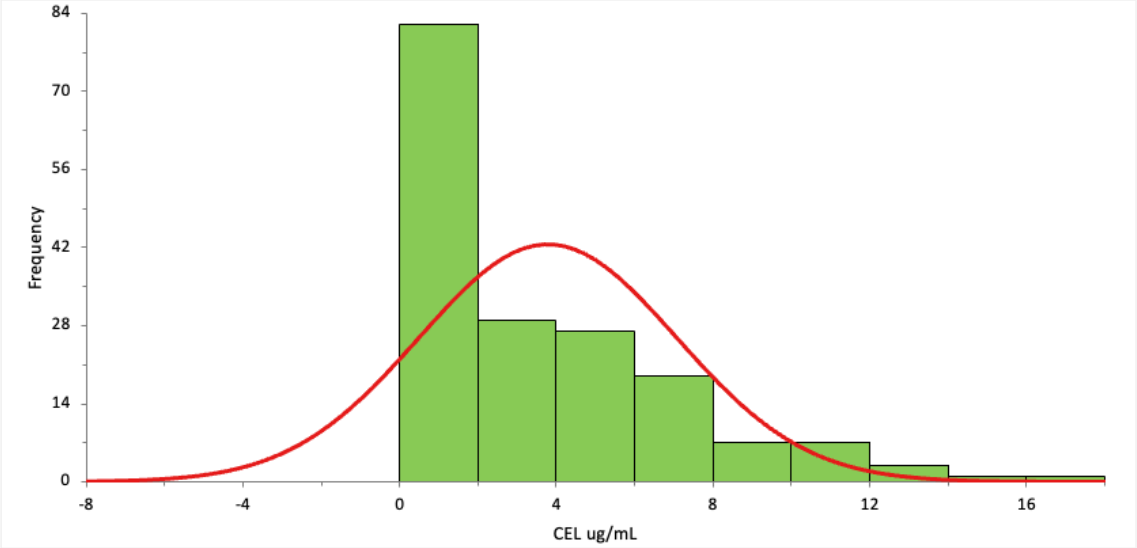
H0: θ = 0
The shift in location between the distributions of the populations is equal to 0.
H1: θ ≠ 0
The shift in location between the distributions of the populations is not equal to 0.
[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Distribution: CEL ug/mL

histogram of Cel data all dogs at all time points with both replicates

Descriptives

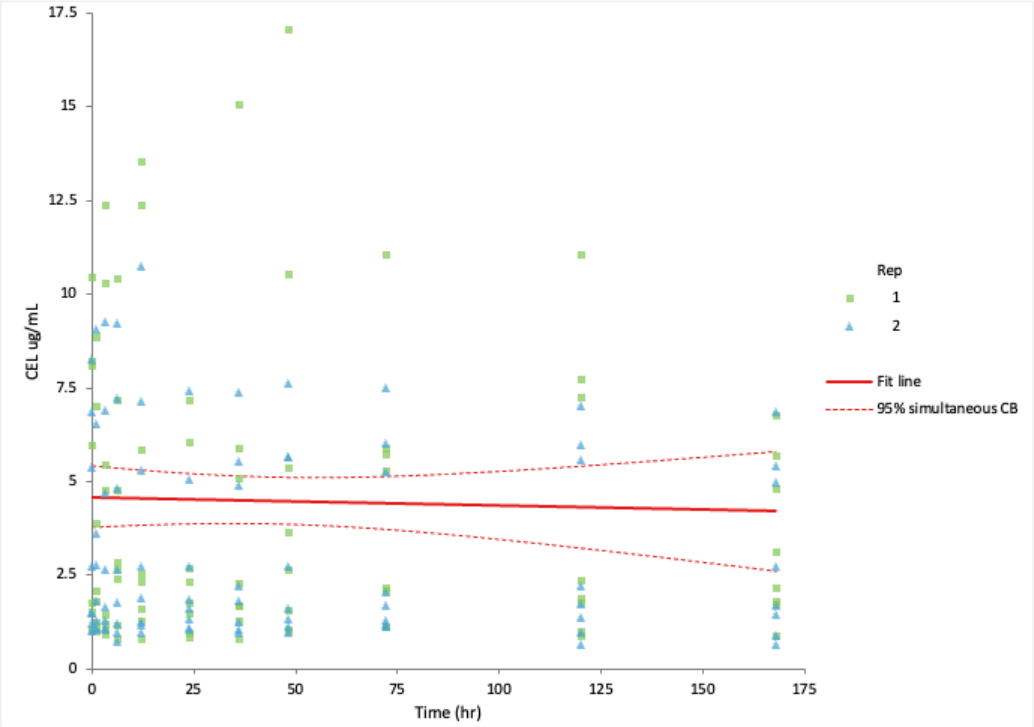


Appendix B

Fit: CEL ug/mL

CEL - linear model fit - all dogs at all time points with both replicates (showing replicates as differentcolors) - can change this to show individual dogs

No time effect, ressiduals are not significantly different.



Fit

N	176
Mean of Y	4.4931
Equation	CEL ug/mL = 4.598 - 0.00236 Time (hr)
Weight	Dog ID
R^2	0.001
R^2 adjusted	-0.004
RMSE	7.16402

Parameter	Estimate	95% CI	SE	t	p-value
Constant	4.598	3.939 to 5.257	0.33378	13.78	<0.0001[1]
Time	-0.002360	-0.01193 to 0.007206	4.8466E-03	-0.49	0.6269[2]

H0: β = 0
The parameter is equal to 0.
H1: β ≠ 0
The parameter is not equal to 0.

[1] Reject the null hypothesis in favour of the alternative hypothesis at the 5% significance level.

[2] Do not reject the null hypothesis at the 5% significance level.

Appendix B

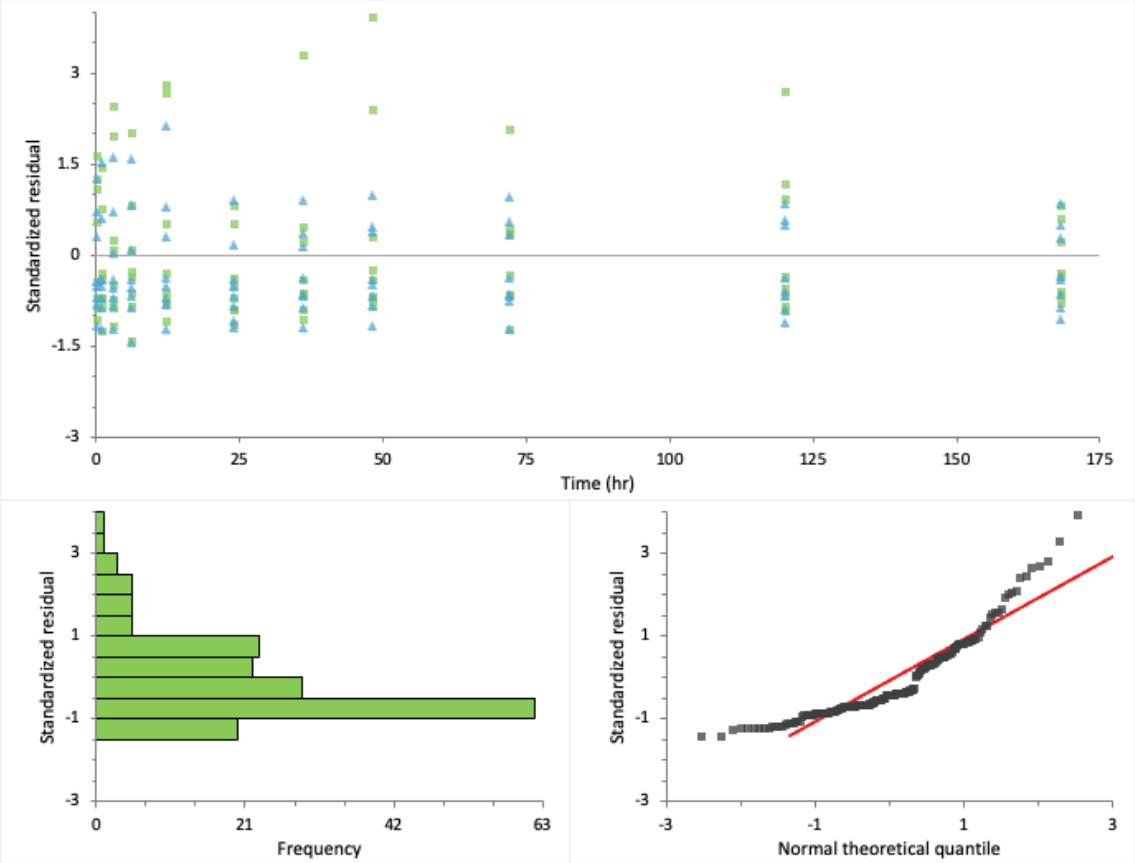
Fit: CEL ug/mL (cont.)

Effect of Model

Source	SS	DF	MS	F	p-value
Difference	12.1707	1	12.1707	0.24	0.6269[1]
Error	8930.2393	174	51.3232		
Null model	8942.4100	175	51.0995		

H0: $E(Y|X=x) = \mu$
The model is no better than a null model $Y=\mu$.
H1: $E(Y|X=x) = \alpha + \beta x$
The model is better than the null model.
[1] Do not reject the null hypothesis at the 5% significance level.

Residuals



Normality

Shapiro-Wilk test

W statistic	0.87
p-value	<0.0001[1]

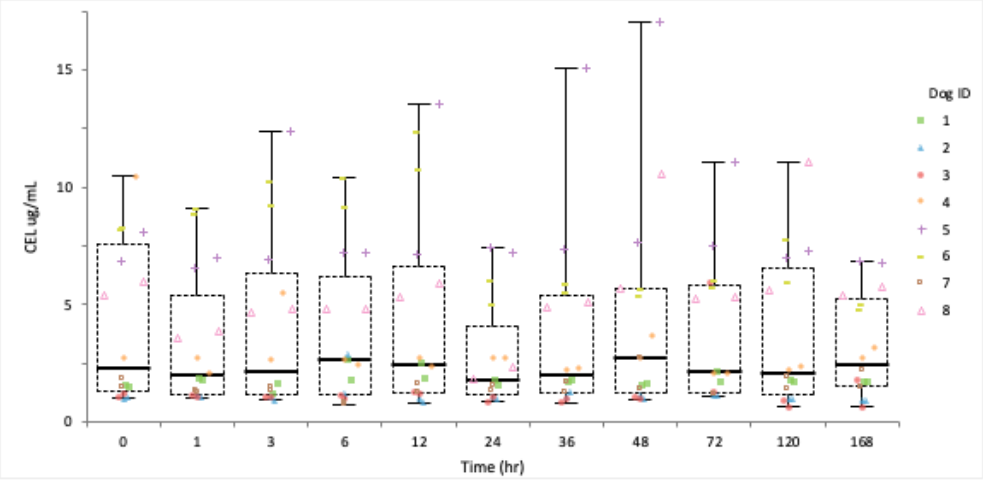
H0: $F(e) = N(\mu, \sigma)$
The distribution of the population is normal with unspecified mean and standard deviation.
H1: $F(e) \neq N(\mu, \sigma)$
The distribution of the population is not normal.
[1] Reject the null hypothesis in favour of the alternative hypothesis at the 5% significance level.

Appendix B

Comparison: CEL ug/mL by Time

CEL box plots - black line is median and dogs are colored individually. No effect on time. Pairwise comparison between time points are not significant

Descriptives



N	176

CEL ug/mL by Time (hr)	Minimum	1st Quartile	Median	95% CI	3rd Quartile	Maximum
0	1.006	1.3156	2.2617	1.2020 to 8.0975	7.5786	10.484
1	1.032	1.1730	1.9672	1.1098 to 6.5243	5.4240	9.084
3	0.943	1.1321	2.1703	1.0838 to 6.9093	6.3110	12.406
6	0.745	1.1759	2.6539	1.1581 to 7.1785	6.1952	10.448
12	0.808	1.2710	2.4405	1.2646 to 7.1441	6.6118	13.539
24	0.866	1.1906	1.7989	1.0853 to 5.0591	4.0979	7.427
36	0.820	1.2721	2.0069	1.2665 to 5.5483	5.3587	15.094
48	0.973	1.2263	2.7002	1.1359 to 5.6742	5.6648	17.065
72	1.120	1.2137	2.1248	1.1671 to 5.9080	5.8331	11.067
120	0.633	1.1624	2.0477	1.0089 to 7.0117	6.5850	11.075
168	0.633	1.5524	2.4659	1.4393 to 5.4188	5.2436	6.880

Quantiles	0.05	0.95
0	1.0105	10.1519
1	1.0379	9.0485
3	0.9576	12.0902
6	0.7532	10.2627
12	0.8295	13.3667
24	0.8832	7.3900
36	0.8448	13.9383
48	0.9760	16.0896
72	1.1207	10.5321
120	0.6743	10.5775
168	0.6719	6.8671

Appendix B

Comparison: CEL ug/mL by Time (cont.)

Location

Kruskal-Wallis test

CEL ug/mL by Time (hr)	N	Rank sum	Mean rank
0	16	1514.0	94.63
1	16	1385.0	86.56
3	16	1401.0	87.56
6	16	1428.0	89.25
12	16	1506.5	94.16
24	16	1262.5	78.91
36	16	1358.0	84.88
48	16	1465.0	91.56
72	16	1490.0	93.13
120	16	1397.5	87.34
168	16	1368.5	85.53

H statistic	1.36
X^2 approximation	1.36
DF	10
p-value	0.9993

H0: $\theta_1 = \theta_2 = \theta...$

The median of the populations are all equal.

H1: $\theta_i \neq \theta_j$ for at least one i,j

The median of the populations are not all equal.

[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Comparison: CEL ug/mL by Time (cont.)

Multiple Comparisons

Steel-Dwass-Critchlow-Fligner all pairs comparisons

Contrast	Hodges-Lehmann location shift	Simultaneous 95% CI	0	p-value
0 - 24	0.4097	-1.6820 to 5.9125		0.9987
6 - 24	0.3349	-2.3993 to 5.3725		1.0000
168 - 24	0.3287	-2.3044 to 3.9171		1.0000
12 - 24	0.3062	-1.9447 to 6.2784		0.9995
48 - 24	0.2999	-1.7796 to 4.8085		0.9998
72 - 24	0.2717	-1.6323 to 4.4443		0.9987
0 - 36	0.2398	-4.0229 to 5.8081		1.0000
12 - 120	0.2300	-4.7328 to 5.7668		1.0000
12 - 36	0.2142	-3.9126 to 6.0870		1.0000
12 - 3	0.1903	-4.6657 to 5.8431		1.0000
12 - 1	0.1832	-3.5354 to 6.0343		1.0000
0 - 1	0.1746	-2.8460 to 5.7437		1.0000
48 - 168	0.1742	-3.4604 to 4.7847		1.0000
1 - 24	0.1713	-3.2313 to 4.7111		1.0000
72 - 36	0.1713	-3.8235 to 4.3427		1.0000
6 - 120	0.1675	-4.7712 to 4.4604		1.0000
0 - 120	0.1662	-4.5352 to 5.8666		1.0000
48 - 36	0.1640	-3.9243 to 4.6785		1.0000
120 - 24	0.1610	-2.0624 to 5.1218		1.0000
48 - 6	0.1569	-3.8460 to 4.7719		1.0000
3 - 24	0.1487	-1.8093 to 5.8240		1.0000
72 - 120	0.1484	-4.8631 to 4.3373		1.0000
6 - 168	0.1397	-3.6122 to 4.4238		1.0000
6 - 36	0.1307	-4.0955 to 4.8996		1.0000
6 - 1	0.1275	-3.8737 to 5.0719		1.0000
0 - 3	0.1135	-4.4030 to 5.5568		1.0000
36 - 24	0.1037	-2.3335 to 4.1195		1.0000
48 - 120	0.0952	-4.6001 to 4.7375		1.0000
72 - 1	0.0899	-3.1756 to 4.2777		1.0000
12 - 72	0.0843	-4.2898 to 5.9770		1.0000
120 - 36	0.0797	-4.0843 to 5.0573		1.0000
48 - 1	0.0787	-3.4094 to 4.5818		1.0000
0 - 72	0.0767	-4.2397 to 5.2415		1.0000
12 - 0	0.0691	-5.5650 to 5.3834		1.0000
48 - 3	0.0569	-4.7749 to 4.6114		1.0000
168 - 36	0.0451	-4.0075 to 3.7319		1.0000
3 - 120	0.0430	-4.7878 to 5.0300		1.0000
3 - 1	0.0323	-3.3761 to 5.6143		1.0000
3 - 36	-0.0031	-4.0358 to 5.2289		1.0000
168 - 1	-0.0073	-3.8511 to 3.7046		1.0000
6 - 3	-0.0176	-5.1256 to 3.8757		1.0000
36 - 1	-0.0185	-3.9904 to 4.0234		1.0000
48 - 12	-0.0268	-5.7914 to 4.5632		1.0000
120 - 1	-0.0419	-3.2766 to 4.9051		1.0000
168 - 3	-0.0472	-5.3045 to 3.7498		1.0000
48 - 0	-0.0639	-5.3643 to 4.5779		1.0000
48 - 72	-0.0748	-4.3154 to 4.5316		1.0000
168 - 120	-0.0905	-4.9394 to 3.7000		1.0000
6 - 12	-0.1001	-5.9433 to 4.4261		1.0000
3 - 72	-0.1014	-4.3670 to 4.8496		1.0000
6 - 72	-0.1336	-4.3361 to 3.9700		1.0000
6 - 0	-0.2161	-5.5711 to 3.7766		1.0000
168 - 72	-0.2629	-4.3074 to 3.5508		1.0000
168 - 12	-0.2714	-6.2545 to 3.5485		1.0000
168 - 0	-0.3331	-5.4129 to 3.6111		1.0000

H0: θ = 0

The shift in location between the distributions of the populations is equal to 0.

H1: θ ≠ 0

The shift in location between the distributions of the populations is not equal to 0.

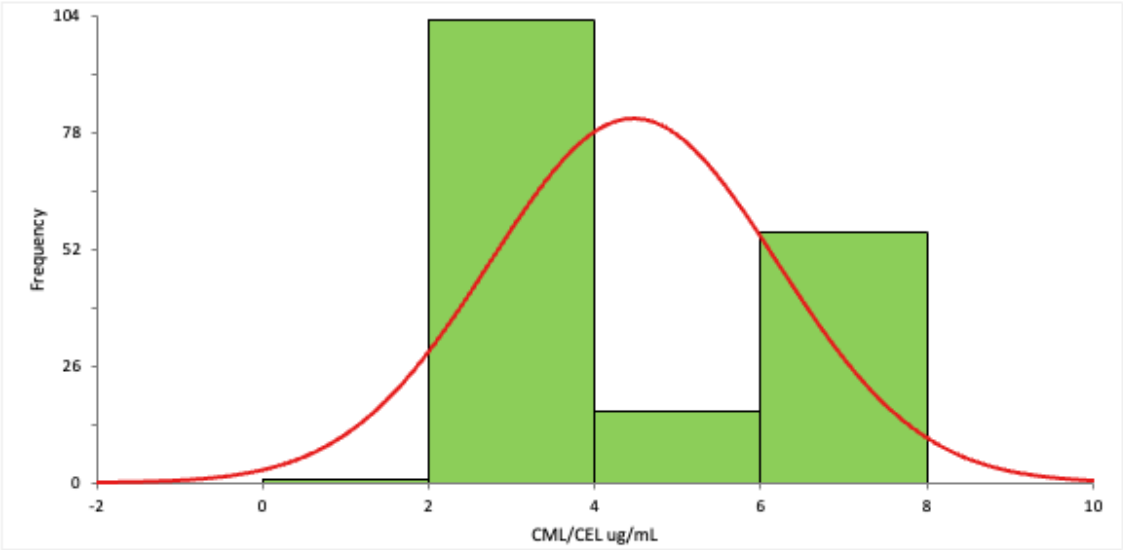
[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Distribution: CML/CEL ug/mL

histogram of CML/CEL data all dogs at all time points with both replicates

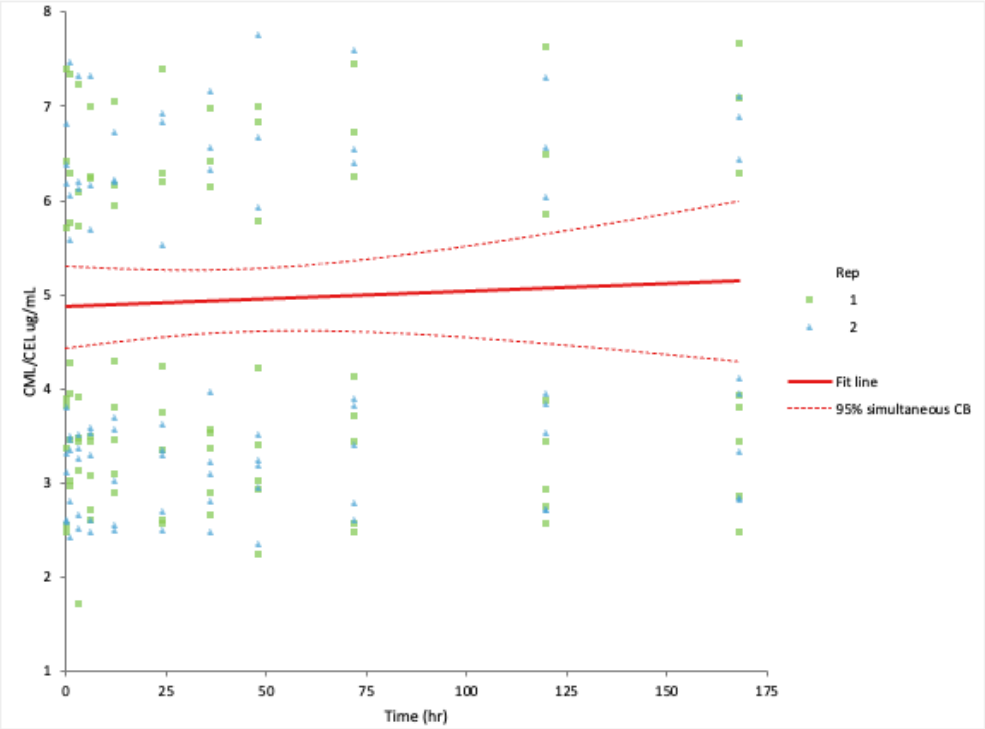
Descriptives



Appendix B

Fit: CML/CEL ug/mL

CML/Cel - linear model fit - all dogs at all time points with both replicates (showing replicates as differentcolors) - can change this to show individual dogs
No time effect, ressiduals are not significantly different.



Fit

N	176
Mean of Y	4.9406
Equation	CML/CEL ug/mL = 4.868 + 0.001638 Time (hr)
Weight	Dog ID
R^2	0.002
R^2 adjusted	-0.003
RMSE	3.81009

Parameter	Estimate	95% CI	SE	t	p-value
Constant	4.868	4.517 to 5.218	0.17752	27.42	<0.0001[1]
Time	0.001638	-0.003449 to 0.006725	2.5776E-03	0.64	0.5259[2]

H0: β = 0
The parameter is equal to 0.
H1: β ≠ 0
The parameter is not equal to 0.
[1] Reject the null hypothesis in favour of the alternative hypothesis at the 5% significance level.
[2] Do not reject the null hypothesis at the 5% significance level.

Appendix B

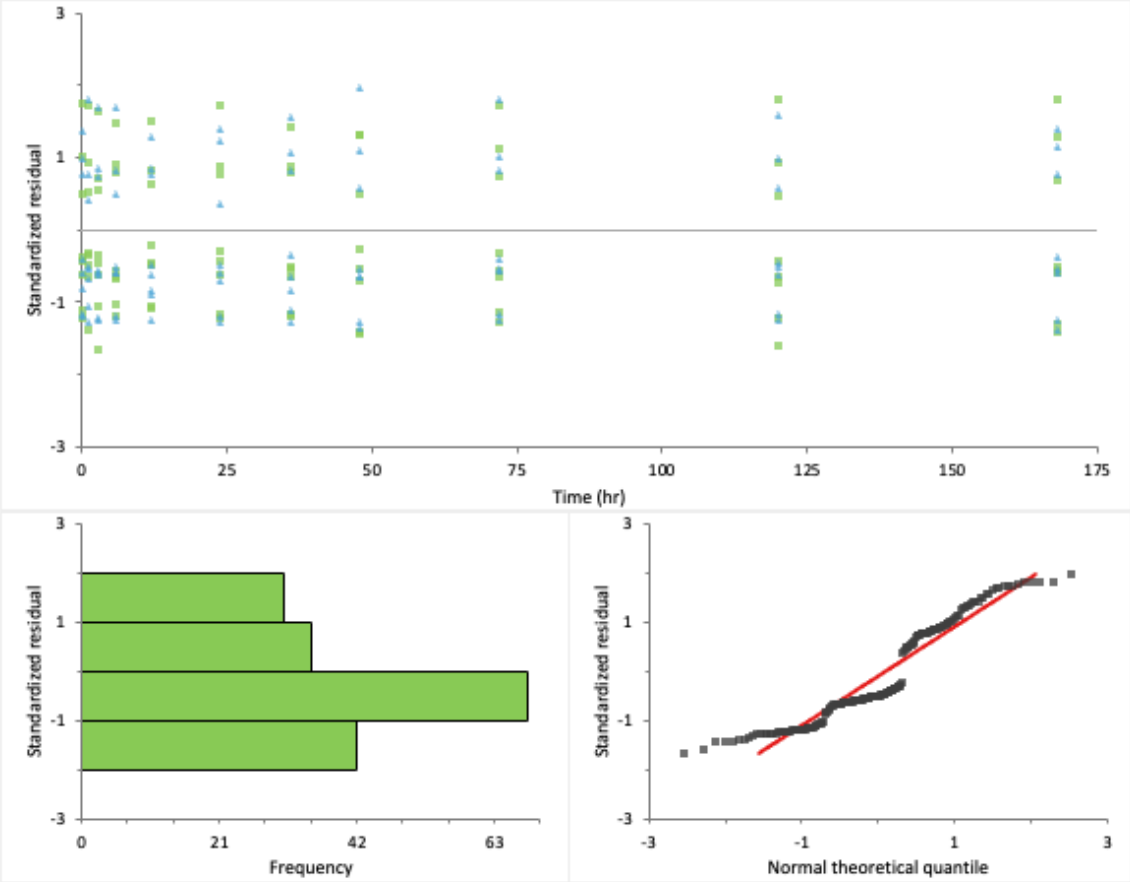
Fit: CML/CEL ug/mL (cont.)

Effect of Model

Source	SS	DF	MS	F	p-value
Difference	5.8628	1	5.8628	0.40	0.5259[1]
Error	2525.9200	174	14.5168		
Null model	2531.7828	175	14.4673		

H0: $E(Y|X=x) = \mu$
The model is no better than a null model $Y=\mu$.
H1: $E(Y|X=x) = \alpha + \beta x$
The model is better than the null model.
[1] Do not reject the null hypothesis at the 5% significance level.

Residuals



Normality

Shapiro-Wilk test

W statistic	0.90
p-value	<0.0001[1]

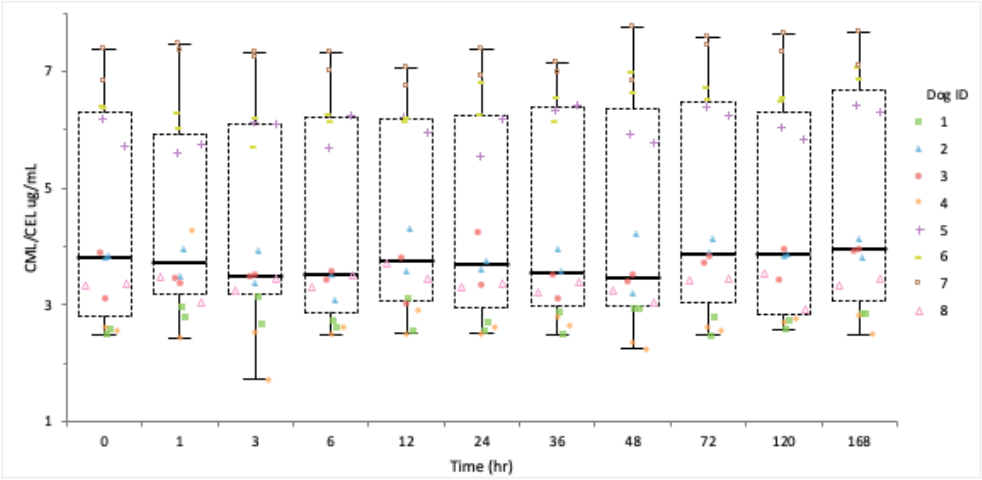
H0: $F(e) = N(\mu, \sigma)$
The distribution of the population is normal with unspecified mean and standard deviation.
H1: $F(e) \neq N(\mu, \sigma)$
The distribution of the population is not normal.
[1] Reject the null hypothesis in favour of the alternative hypothesis at the 5% significance level.

Appendix B

Comparison: CML/CEL ug/mL by Time

CML/CEL box plots - black line is median and dogs are colored individually. No effect on time. Pairwise comparison between time points are not significant

Descriptives



N	176

CML/CEL ug/mL by Time (hr)	Minimum	1st Quartile	Median	95% CI	3rd Quartile	Maximum
0	2.490	2.8158	3.8156	2.6043 to 6.3888	6.3025	7.390
1	2.433	3.1706	3.7251	3.0344 to 6.0469	5.9286	7.469
3	1.725	3.1879	3.4973	3.1391 to 6.1238	6.1090	7.318
6	2.486	2.8673	3.5169	2.7198 to 6.2451	6.2131	7.322
12	2.506	3.0572	3.7546	3.0209 to 6.2055	6.1879	7.051
24	2.506	2.9445	3.6890	2.6921 to 6.2864	6.2472	7.395
36	2.491	2.9798	3.5558	2.8925 to 6.4267	6.3820	7.156
48	2.252	2.9823	3.4652	2.9452 to 6.6659	6.3617	7.761
72	2.485	3.0462	3.8671	2.7882 to 6.5405	6.4829	7.588
120	2.580	2.8339	3.8620	2.7628 to 6.4958	6.3024	7.638
168	2.485	3.0598	3.9435	2.8626 to 6.8853	6.6956	7.676

Quantiles	0.05	0.95
0	2.4991	7.3055
1	2.4884	7.4514
3	1.8450	7.3045
6	2.5045	7.2728
12	2.5126	7.0033
24	2.5153	7.3248
36	2.5162	7.1304
48	2.2673	7.6474
72	2.4974	7.5666
120	2.5994	7.5893
168	2.5372	7.5893

Appendix B

Comparison: CML/CEL ug/mL by Time (cont.)

Location

Kruskal-Wallis test

CML/CEL ug/mL by Time (hr)	N	Rank sum	Mean rank
0	16	1353.0	84.56
1	16	1445.0	90.31
3	16	1349.0	84.31
6	16	1322.0	82.63
12	16	1417.5	88.59
24	16	1386.5	86.66
36	16	1410.0	88.13
48	16	1359.0	84.94
72	16	1509.0	94.31
120	16	1456.5	91.03
168	16	1568.5	98.03

H statistic	1.34
X^2 approximation	1.34
DF	10
p-value	0.9993

H0: $\theta_1 = \theta_2 = \theta...$

The median of the populations are all equal.

H1: $\theta_i \neq \theta_j$ for at least one i,j

The median of the populations are not all equal.

[1] Do not reject the null hypothesis at the 5% significance level.

Appendix B

Comparison: CML/CEL ug/mL by Time (cont.)

Multiple Comparisons

Steel-Dwass-Critchlow-Fligner all pairs comparisons

Contrast	Hodges-Lehmann location shift	Simultaneous 95% CI	0	p-value
168 - 6	0.3487	-2.7309 to 3.5677		0.9974
168 - 3	0.3134	-2.6508 to 3.4023		0.9999
168 - 48	0.2939	-2.8846 to 3.4590		1.0000
72 - 3	0.2656	-2.6403 to 3.1692		0.9999
168 - 0	0.2642	-2.7058 to 3.2911		0.9994
72 - 48	0.2597	-2.9509 to 3.3076		1.0000
72 - 6	0.2563	-2.7204 to 3.2454		0.9998
168 - 12	0.2407	-2.6181 to 3.2940		1.0000
168 - 24	0.2307	-2.7548 to 3.3419		0.9999
168 - 36	0.2176	-2.8631 to 3.4860		1.0000
120 - 6	0.1841	-2.7658 to 3.0586		1.0000
168 - 1	0.1527	-2.4271 to 3.2559		1.0000
72 - 36	0.1465	-2.8560 to 3.3001		1.0000
72 - 12	0.1340	-2.5904 to 3.1449		1.0000
168 - 120	0.1320	-2.6959 to 3.2567		1.0000
120 - 0	0.1224	-2.8739 to 3.1291		1.0000
72 - 0	0.1209	-2.7072 to 3.2241		1.0000
120 - 24	0.1194	-2.8447 to 3.0762		1.0000
120 - 3	0.1127	-2.6822 to 3.0506		1.0000
72 - 1	0.1121	-2.4673 to 3.0790		1.0000
1 - 6	0.1056	-2.7428 to 2.7684		1.0000
1 - 24	0.1038	-2.7840 to 2.6888		1.0000
24 - 48	0.1038	-3.0981 to 3.0333		1.0000
72 - 24	0.1018	-2.7849 to 3.1824		1.0000
1 - 48	0.0908	-2.8862 to 2.7794		1.0000
1 - 36	0.0873	-2.9583 to 2.7176		1.0000
3 - 48	0.0841	-3.1542 to 2.8708		1.0000
12 - 3	0.0825	-2.6335 to 2.7921		1.0000
168 - 72	0.0810	-2.9647 to 3.1908		1.0000
12 - 48	0.0806	-2.9149 to 2.9524		1.0000
36 - 6	0.0772	-2.8853 to 3.1317		1.0000
120 - 48	0.0765	-3.0273 to 3.0826		1.0000
1 - 3	0.0737	-2.6854 to 2.6237		1.0000
24 - 3	0.0730	-2.7682 to 2.9182		1.0000
12 - 6	0.0671	-2.6871 to 2.8799		1.0000
120 - 36	0.0575	-3.0312 to 3.0306		1.0000
36 - 3	0.0549	-2.8279 to 3.0592		1.0000
24 - 6	0.0509	-2.8705 to 2.9913		1.0000
36 - 48	0.0509	-3.0870 to 3.1577		1.0000
120 - 12	0.0355	-2.7638 to 2.9224		1.0000
12 - 1	0.0339	-2.5922 to 2.7407		1.0000
0 - 6	0.0297	-2.8939 to 2.9849		1.0000
0 - 48	0.0273	-3.0990 to 2.9948		1.0000
72 - 120	0.0250	-2.7809 to 3.1921		1.0000
6 - 48	0.0175	-3.2160 to 2.9153		1.0000
0 - 3	0.0113	-2.8074 to 2.9254		1.0000
12 - 36	0.0110	-2.8681 to 2.8518		1.0000
6 - 3	0.0106	-2.7932 to 2.7999		1.0000
12 - 24	0.0066	-2.7375 to 2.8123		1.0000
0 - 12	-0.0009	-2.8470 to 2.7269		1.0000
0 - 24	-0.0130	-2.9700 to 2.8838		1.0000
24 - 36	-0.0184	-2.9638 to 2.9646		1.0000
120 - 1	-0.0215	-2.6577 to 2.9935		1.0000
0 - 36	-0.0576	-3.0775 to 2.9109		1.0000
0 - 1	-0.1065	-2.6803 to 2.8205		1.0000

H0: θ = 0

The shift in location between the distributions of the populations is equal to 0.

H1: θ ≠ 0

The shift in location between the distributions of the populations is not equal to 0.

[1] Do not reject the null hypothesis at the 5% significance level.