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A novel canine nuclear magnetic resonance spectroscopy-based metabolomics platform: Validation and sample handling

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Abstract

Background: Metabolomics has been proven to be an invaluable research tool by providing comprehensive insight into systemic metabolism. However, the lack of scalable and quantitative methods with known reference intervals (RIs) and documented reproducibility has prevented the use of metabolomics in the clinical setting.

Objective: The objective of this study was to validate the developed quantitative nuclear magnetic resonance (NMR) spectroscopy-based metabolomics platform for canine serum and plasma samples and determine optimal sample handling conditions for its use.

Methods: Altogether, 8247 canine samples were analyzed using a Bruker's 500 MHz NMR spectrometer. Using statistical approaches derived from international guidelines, we studied method precision, measurand stability in various long- and short-term storage conditions, as well as the effect of prolonged contact with red blood cells (RBCs), and differences among blood collection tubes. We also screened interferences with lipemia, hemolysis, and bilirubinemia. The results were compared against routine clinical chemistry methods, and RIs were defined for all measurands.

Results: We determined RIs for 123 measurands, most of which were previously unpublished. The reproducibility of the results of the NMR platform appeared generally outstanding, and the integrity of the results can be ensured by following standard blood drawing and processing guidelines.

Conclusions: Owing to the advantages of quantitative results, high reproducibility, and scalability, this canine metabolomics platform holds great potential for numerous clinical and research applications to improve canine health and well-being.

KEYWORDS

dog, precision, reference intervals, stability, storage

1 | INTRODUCTION

Metabolomics is an omics-based approach that generates comprehensive information of metabolism, enabling an extensive view on the current state of system metabolism. Metabolomics has become increasingly popular in canine studies. It has been used to characterize the metabolic effects of multiple environmental factors, and inter- and intra-individual factors, including feeding, aging, interbreed differences, drug action, behavior, exercise, genetic factors, and pathologic processes.[1]

Mass spectrometry (MS) and proton nuclear magnetic resonance (NMR) spectroscopy are the two main metabolomics technologies.[2] NMR spectroscopy offers cost-effectively quantitative data with high reproducibility, high throughput, and excellent scalability.[2] NMR spectroscopy is well suited for the scientific use of large cohorts and biobanks,[3,4] and is highly suited for studies combining different omics technologies.[4,5] Due to the quantitative and highly reproducible nature of NMR spectroscopy, this technique can be easily used as a diagnostic tool, offering research findings to be easily applied for clinical use.

Metabolomics holds great potential for clinical diagnostics. It is proving to be valuable for disease risk predictions.[6-10] For example, in human type 2 diabetes mellitus, metabolic changes have been found to occur years before disease onset, enabling supportive and preventive measures before clinical manifestations occur.[10] Metabolomics is also viewed as a potential tool for the evaluation of disease severity and differential diagnostics.[10,11] The metabolic state of an individual might also impact the best treatment of a patient, affecting drug responses, drug toxicities, treatment efficacies, and nutritional needs.[11-13] Especially, the management of chronic metabolic diseases is thought to benefit from a method capable of generating comprehensive information on the metabolic state of an individual, enabling a personalized approach to disease management.[10] In contrast to genetics, metabolomics offers real-time information about the metabolic state of an animal, taking environmental factors and treatments into account. This offers metabolomics the ability to be used as both a diagnostic and follow-up tool.

Prerequisites for the utility of a particular method include the documentation of method reproducibility, an understanding regarding the factors influencing the results, and the formation of reference intervals (RIs).[14,15] All of these factors remain largely unpublished in the canine metabolomics field. The objectives of this study were to conduct the method validation studies most appropriate for the clinical use of the method and determine the most appropriate sample handling practices for its use. To achieve this, we determined analytic precision and RIs for all measurands. We also, conducted an interference screen and a method comparison study, and studied storage stability, the effects of prolonged contact with red blood cells, and the differences between sample tubes.

2 | MATERIALS AND METHODS

2.1 | Proton NMR spectroscopy

The (NMR) metabolomics technique is similar to the technique used in human samples.[16] The highly automated process uses a Bruker AVANCE III HD 500 spectrometer with a 5 mm triple-channel (1H, 13C, 15N) z-gradient Prodigy probe head (Bruker Biospin) capable of quantifying 200 samples every 24 hours. Usable sample types are serum, EDTA plasma, heparinized plasma, and citrated plasma.

The laboratory that performed the NMR analyses complied with the EN ISO 13485:2016, SFS-EN ISO/IEC 17025:2017, and SFS-EN ISO 15189:2013 standards.[17-19] It has several quality assurance processes to ensure high-quality results, including constant performance testing of analytic equipment, monitoring sample preparation, and assessing quality using quality control (QC) samples. Two plasma QC samples are included in each 96-sample rack, and a technical control sample is profiled with each NMR instrument at regular intervals. The control sample data are reviewed and reported periodically. Furthermore, automated data processing procedures check for unexpected signals and irregularities. Integrated procedures verify the quality of each sample by reporting signs of sample degradation and contamination. If measurand levels are below the limit of detection, the value is presented as missing. Since the entire testing process is highly automated, operator-to-operator variability can be regarded as negligible.

To develop, validate, and use this method in dogs, the canine NMR metabolomics project was initiated. A total of 8247 serum or plasma samples were analyzed from fall 2016 to fall 2018 (Figure S1), with samples obtained from 6164 individual client-owned dogs (Table 1). Most samples were collected by cephalic venipuncture during organized sampling times; 439 samples were collected at Finnish veterinary clinics and sent to this project by mail as EDTA-anticoagulated whole blood; 999 samples were routine diagnostic material from a veterinary laboratory. Samples were frozen at −80°C after plasma/serum separation within 15 minutes and 240 hours of collection and stored there for 1 week to 1 year before NMR analysis. For all purposefully collected samples, the owners completed a history form, including the dog's signalment and details about the health status, diet, exercise, stress, and reproductive state.

Initial method calibration was conducted using 847 EDTA plasma samples and 120 serum samples. The characteristic NMR signals of amino acids, glycolysis-related metabolites, creatinine, and glycoprotein acetyls (GlycA) are well-known, and the quantitative and linear relationship between signal intensities and molecular concentrations is an inherent property of NMR. Thus, these measurands can be quantified using the established NMR techniques. The signal identification of triglycerides, cholesterol, and lipoproteins was verified and calibrated by analyzing 200 EDTA plasma samples with different lipid concentrations using high-performance liquid chromatography,[20] and fatty acids were similarly analyzed with 100 samples in a commercial laboratory (Vitas as, Norway) using a chromatographic method.

TABLE 1 Demographics of the dogs in this study

Dogs, n		Breeds, n	Males%/females%	Puppies % (n)	Adults % (n)	Seniors % (n)
All[a,b]	6164	256	40/60	18 (1094)	61 (3764)	21 (1303)
Serum[a]	4816	223	40/60	18 (886)	60 (2871)	22 (1057)
Heparinized plasma	498	117	40/60	13 (67)	63 (315)	23 (116)
1031		158	42/58	15 (153)	67 (686)	18 (191)

Note: Demographics of individual client-owned dogs with samples collected during the canine NMR metabolomics project. Some individuals had replicate samples taken or samples of multiple sample types. The table does not include the leftover clinical laboratory samples used in the method comparison study. Puppies were aged <1 year, adults 1-7 years and seniors >7 years.

[a]The ages of two dogs were unknown in the serum group.

[b]The age of one dog was unknown in the EDTA plasma group.

2.2 | Precision

The precision of the NMR platform was evaluated using the one-run-per-day procedure from Clinical & Laboratory Standards Institute (CLSI) EP5-A3.[21] Since it was not feasible to collect samples at clinical decision limits for all measurands, we chose to use three dogs in different physiologic states (puppy, adult, and senior dog suffering from hyperadrenocorticism). Blood was drawn by cephalic venipuncture into Vacuette 8 ml Z Serum Sep Clot Activator tubes (Greiner Bio-One). After clotting for 30-45 minutes, the samples were centrifuged at 2000*g* for 10 minutes to separate serum. Every sample was divided into 40 aliquots of 100 μL and stored at −80°C for 1 week to 2 months. Two duplicate aliquots of each sample were analyzed each day during a 20-day time period.[21]

The sample from the senior dog suffering from hyperadrenocorticism showed marked chylomicronemia and was used to evaluate the effect of chylomicronemia on analytical precision.

Total within-laboratory precision estimates were expressed as both the coefficient of variation (CV%) and standard deviation (S_T). Within-run precision was expressed as the within-run standard deviation (S_r). Combined between-day and between-run precision were expressed as the standard deviation of the daily means (B). Outlier detection was based on the differences in the duplicate aliquots in the same run compared with the mean difference of all duplicates from the sample. A difference that exceeded the mean difference fourfold was set as the rejection line for the exclusion of the duplicates.

The most advisable hierarchy for performance goals in human diagnostic laboratories include (1) biological outcomes, (2) biological variation, and (3) state-of-the-art goals.[22,23] Biological outcome-based performance goals are yet unpublished for animals, and both biological variation- and state-of-the-art goals have their own pitfalls. Thus, we evaluated the precision estimates against a set of precision goals:

1. Biological variation-based CV% goals (CV_{BV}).[24] These goals have drawbacks in that they can be too strict, unachievable by clinical laboratories, and do not affect result interpretations.[25]
2. Total allowable error (TE_a) goals based on clinical decision limits (CV_{CDL}). The total error of veterinary laboratory results is evaluated using observed total error (TE_{obs}), which is then evaluated against preset TE_a limits. TE_{obs} can be calculated as TE_{obs} = Bias% + 2 CV%. Bias was not applicable for this study as a deviation from a true value.[25] This led us to use ½ of the American Society for Veterinary Clinical Pathology (ASVCP) TE_a limits based on CV_{CDLs} as a precision goal for the CV%.
3. For measurands where biological variation- or TE_a-based goals were unavailable, we used 20% as the CV% goal (CV_{BG}), which is generally considered an acceptable error in metabolomics.[26,27] However, this goal might not be descriptive for measurands with very wide or narrow reference intervals caused by inter-individual biological variation.
4. A comparison of the S_T to the S_T goal of under 1/8 reference widths (S_{Tmax}). This goal was set since analytical variation over one-quarter of the reference interval could affect clinical interpretation.[28] However, this goal has a drawback in that imprecision affects the width of the reference interval.[29]

2.3 | Interference screen

We performed interference screens for hemolysis, lipemia, and unconjugated and conjugated bilirubin with a protocol derived from CLSI-EP7-A2.[30] Interference was evaluated on primary measurands, not ratio and percentage measurands. Lipemia interference was only evaluated for molecules other than lipids since the lipid removal process inevitably decreases lipid concentration.

For hemolysis interference screening, 5 mL of heparinized blood was collected from two client-owned dogs by cephalic venipuncture. The samples were centrifuged, and plasma was separated and discarded. Hemolysates were prepared from the red blood cells using the osmotic shock procedure, and hemoglobin concentrations were measured using an ADVIA 2120i hematology analyzer (Siemens).[31] The base of the test and control samples was created by collecting 5 mL heparinized blood by cephalic venipuncture from the same dogs. To produce test samples containing 500 mg/dL hemoglobin, a measured volume of hemolysate from one dog was added to the plasma of the same dog.

Bilirubin interference was screened by creating two sample pools with measurand concentrations that were as different as possible. Baseline bilirubin concentrations were measured (540 nm) using a modified version of the acid diazo coupling (Malloy-Evelyn) method (Bilirubin Total (NBD), Thermo Fisher Scientific) and a Konelab 60i chemistry analyzer (Thermo Fisher Scientific).[32] The two pools were divided into four pools, creating the bases for separate test and control pools for unconjugated and conjugated bilirubin. The test pools for conjugated bilirubin were prepared by adding the 201102 EMD Millipore bilirubin conjugate (Sigma-Aldrich), and unconjugated bilirubin was prepared by adding 2011 EMD Millipore bilirubin (Sigma-Aldrich). All pools were in 20 mg/dL concentrations. The control pools for conjugated and unconjugated bilirubin were prepared by adding the same volume of water or NaOH, respectively.

Lipemia interference was screened by pooling lipemic serum samples to create a pool with a triglyceride concentration around 2 mmol/L. The samples had been previously stored at −80°C for 1-2 years. The created pools were divided into test and control pools, and lipids in the control pool were cleared by ultracentrifugation.

All resulting samples/pools were divided into triplicate aliquots, and could detect interference effects over 3.0× within-run precision with 95% confidence and power.[30] The bilirubin interference was analyzed in samples within a day whereas hemolytic samples were stored at −80°C for 2 days, and lipemia samples were stored for 2 weeks before NMR analysis.

Statistical testing was based on point estimate comparisons of the observed interference effect (d_{obs}) and the cutoff value (d_c), according to CLSI-EP7-A2.[30]

The interference effect, $d_{obs,}$ was defined as the difference between the means of the test and control samples:

$$d_{obs} = \bar{x}_{test} - \bar{x}_{control}$$

The cutoff value, d_c, was defined as $d_c = \left(d_{null} + sz_{1-\alpha/2}\right)/(\sqrt{n})$, where d_{null} represents the assumed difference between the means of test and control samples (set to 0), n is the number of replicates (n = 3), and s is the mean repeatability standard deviation (S_r) of non-chylomicronemic samples obtained from the precision study. We calculated 95% confidence intervals for d_{obs} as follows:

$$d_{obs} = \bar{x}_{test} - \bar{x}_{control} \pm t_{0,975,n-1} s\sqrt{\frac{2}{n}}.$$

We established our decision of interference on the lower 95% limit of interference cutoff value (d_{obs_low}). Interference was present if $d_{obs\_low} > d_c$ in at least one sample/pool since matrix properties and/or measurand concentrations might affect the presence of interference.

2.4 | Method comparison

The method comparison study was designed to evaluate whether the results of routine clinical chemistry measurands included in the NMR metabolomics platform were interchangeable with the results obtained using a conventional clinical chemistry method. Bias was viewed as a measure of method interchangeability since the values obtained by the conventional method cannot be regarded as true measurand values.

The used samples (n = 999) were routine diagnostic canine sample material submitted by veterinarians across Finland, sent by mail to a single laboratory (Movet Oy). Upon arrival, the samples were divided into two aliquots. One aliquot was immediately analyzed with a Thermo Fischer Scientific Konelab Prime 60i chemistry analyzer (Thermo Fisher Scientific) using automated methods. Triglycerides, cholesterol, and creatinine were measured by enzymatic methods, albumin by a photometric method using bromcresol green, glucose by the IFCC-standard method using hexokinase, and lactate by a colorimetric method using lactate oxidase. QC for the conventional method measurands included two QC sample measurements at different levels before running the samples. For creatinine, only one QC sample was analyzed. The QC procedure was conducted following the QC limits (±2 SD) provided by the control manufacturer.

The other aliquot was frozen and stored at −20°C for a maximum of 4 weeks before shipment for the NMR analysis. The samples were sent in three batches on freezer blocks with a shipping time of 7-14 hours. At the NMR laboratory, the samples were stored at −80°C for a maximum of 1 week before analysis. One of the sample shipments was sent over a 14 h period on freezer blocks and partly thawed during shipment. The NMR albumin measurement was scaled using 404 samples; these samples were excluded from the albumin method comparison.

Statistical analysis followed the ASVCP principles of quality assurance and standards.[15] Comparison plots were created for raw data, logarithm-transformed data, and Deming-transformed data. Deming regression[33,34] was used to calculate the slope and intercept for all measurands and linear regression for measurands with a correlation over 0.99. The mean bias was determined at both ends of the reference interval using the slope and intercept. Bland-Altman plots[35] were used to visualize the distribution of differences. To determine method interchangeability, total observed error (TE_{obs}) was evaluated against ASVCP clinical chemistry guidelines' total allowable error goals (TE_a) based on clinical decision limits,[25] which reflect the maximum values that would affect clinical decision-making.

2.5 | Sample storage study

In the sample storage study, we examined the effects of both long- and short-term sample storage conditions on analytic results. The sample storage study protocol is presented in Table S1. Measurand stability was studied only in primary measurands, not in ratio and percentage measurands.

Serum and EDTA plasma samples from seven client-owned dogs and heparinized plasma samples from four dogs were collected in separate sampling situations. Samples were drawn by cephalic

venipuncture, centrifuged, and separated according to the tube manufacturer's recommendations. The samples were divided into aliquots and stored in the test conditions (Table S1). Serum and EDTA plasma samples were analyzed by NMR immediately after storage in the different test conditions, whereas heparinized plasma samples were additionally stored at −80°C for 2-3 weeks.

We used the two-sided Wilcoxon's exact test to estimate the statistical significance of the changes in measurand values between samples not subjected to storage (T_0) and samples subjected to different storage conditions at each storage time point (T_x) (Table S1). In addition to the Wilcoxon test, we calculated the mean percentage deviation (MPD) using the following formula to indicate the magnitude of the change[36]:

$$\mathrm{MPD} = \left(\frac{T_{x_{mean}} - T_{0_{mean}}}{T_{0_{mean}}} \right) x100,$$

MPD was compared with the acceptable change limit (ACL), according to ISO 5725-6, and calculated using the following formula[37]:

$$\mathrm{ACL} = 2.77\,\mathrm{CVa}$$

The CVa was obtained from the precision study results of this study and calculated as the mean CV% of the nonchylomicronemic samples. The factor 2.77 was derived from 1.96 × √2, where 1.96 represents the 95% confidence interval for bi-directional changes, and √2 was used since we compared two results with the same CVa. An MPD higher than the ACL represents a probable change in measurand concentration.

Outlier detection cut points were defined as:

$$\left(\overline{T_x - T_0}\right) \pm 2 \cdot \mathrm{STD}_{(T_x - T_0)},$$

where $\overline{T_x - T_0}$ is the mean and $\mathrm{STD}_{(\overline{T_x - T_0})}$ is the standard deviation of the difference in measurand results at storage time T_x and collection time T_0.

If the difference $T_{xi} - T_{0i}$ of the sample *i* exceeded or undercut these cut points, it was defined as an outlier. All analyses were performed as both outliers included and excluded to evaluate the effect of outlier exclusion.

The criteria for clinically significant change were set so that the change would be both statistically significant (Wilcoxon $P < 0.05$) and the magnitude of the change, evaluated as MPD, would exceed the ACL. The change was required to be consistent at the remaining time points.

2.6 | Delayed plasma separation study

In the delayed plasma separation study, we defined how 24- and 48-hour delays in plasma separation affect measurand values.

Blood was drawn by cephalic venipuncture from 34 client-owned dogs into three Vacuette K2 EDTA tubes per dog. One of the tubes was centrifuged, and plasma was separated within 1 hour of sampling. The remaining two tubes were stored as whole blood in a refrigerator until centrifugation, and plasma was separated after 24 and 48 hours. After plasma separation, all samples were stored at −80°C for 1-1.5 years before NMR analysis. This experiment was conducted using an older version of the NMR platform, with a lower number of measurands, lacking fatty acids, for example.

Statistical methods were the same as in the sample storage study. T_0 in the delayed plasma study represented plasma separated according to the sample tube manufacturer recommendations, and T_x plasma separated after 24 and 48 hours.

2.7 | Sample tube validation

To study whether different blood collection tubes affect measurand values, we studied differences between different blood collection tubes and their lots. The protocol was modified from the proposed process by Bowen and Adcock[38] and was conducted only for primary measurands, not for ratio and percentage measurands.

Thirty milliliters of blood were drawn from an intravenous cannula in the cephalic vein of 20 dogs into seven different types of blood collection tubes (Table S2), each tube type having two tubes from different lots. All samples were centrifuged and separated according to sample tube manufacturer's recommendations. The centrifugation conditions for the MiniCollect tubes were 3000*g* for 10 minutes and for all other tube types were 2000*g* for 13 minutes. All samples were stored at −80°C for 1-2 months before NMR analysis.

Differences between different sample tubes were tested using this protocol

1. Study where the tubes give comparable results to the primary reference tube; the Vacuette lithium heparin tube was set as the primary reference tube (T_0), and all other tubes (T_x) were compared against it.
2. Study where the serum tubes give comparable results to the serum reference tube (as we hypothesized, that most of the differences between sample tubes would originate from differences between serum and plasma): the Vacuette Z Serum Clot Activator tube was set as the serum reference tube (T_0) and all other serum tubes (T_x) were compared against it.

The average of the measurand values in the two lots was used in these calculations. Lot-to-lot variability was tested using this protocol

1. Compare the two lots of each tube to one another; tubes in lot A were set as the reference tubes (T_0), and tubes in lot B (T_x) were compared against them.

2. If lot-to-lot variability was observed, results of each lot were compared with the reference tubes to determine if samples from both lots would give comparable results to the other tube types.

Lots A and B were compared separately to the Vacuette Lithium Heparin and Vacuette Z Serum Clot Activator tubes.

Similar statistical methods were used as in the sample storage study. For the outlier detection protocol, the coefficient of 3 for the STD (instead of 2 in the sample storage study) was used for cut points: $(\overline{T_x - T_0}) \mp 3 \cdot STD_{(T_x - T_0)}$. We chose this STD since outlier testing was more sensitive due to more observations in this study.

In addition to the statistical methods being similar to the storage study, we calculated bias as the mean difference of T_x and T_0 with 95% confidence intervals to highlight the limits where the differences reside.

Although measurand quantification is possible from citrate tubes, we did not include this tube type in our study, since this tube type is typically not used in clinical applications of this field.

2.8 | Reference intervals

Determination of RIs was performed according to the American College of Veterinary Clinical Pathology (ASVCP) reference interval guidelines.[14] Inclusion in the RI calculation was based on fasting duration (minimum of 12 hours), appropriate sample handling, and the lack of owner-reported biological confounding factors, including diseases, severe anxiety/stress, and strenuous exercise.

We used the population-based nonparametric method for RI calculation to calculate reference limits as 2.5th and 97.5th percentile limits and calculated 90% confidence intervals (CIs) for the reference limits. The lower reference limit, r1, was computed as the observation corresponding to r = 0.025 (n + 1), and the upper reference limit, r2, as the observation corresponding to r = 0.975 (n + 1). When the values of r1 and r2 were not integers, the limits were calculated by interpolating between the data points corresponding to the ranks on either side of r1 and r2. The 90% CIs for the reference limits were assigned according to the published sample size-specific rank numbers.

A total of 865 serum samples collected for the canine NMR metabolomics project were included in the serum RI calculations. The samples were chosen to include over 120 samples of puppies (under 1 year old), adults (1-7 years old), and senior dogs (over 7 years old) for age-specific RI calculations. The 865 samples consisted of individuals from 68 breeds; 347 males and 517 females, with 152 puppies, 545 adult dogs, and 168 senior dogs.

A total of 269 samples out of the initial 495 samples collected for the canine NMR metabolomics project were qualified for lithium heparinized plasma RI determinations. These samples consisted of individuals from 83 breeds; 155 males and 114 females, with 29 puppies, 196 adult dogs, and 44 senior dogs. Age-specific RIs were calculated for the adult dogs.

Each measurand was examined for outliers. We used Box-Cox transformations to find normal transformations and Horn's algorithm in the transformed data to identify outliers. Values were considered outliers if they exceeded interquartile (IQ) fences set at Q1 − 1.5*IQR and Q3 + 1.5*IQR (IQR = interquartile range; IQR = IQ3 − IQ1, where IQ1 and IQ3 are the 25th and 75th percentiles, respectively). After outlier identification, the sample and animal data were thoroughly reviewed for confounding factors before reaching a conclusion about sample exclusion. In measurands with non-normal distributions after data transformation, Horn's algorithm offered multiple outliers, which were all reviewed before reaching a conclusion about exclusion.

Lastly, we studied the relation between 90% CIs of the reference limits and RIs. It is recommended that CIs should not exceed 0.2 times the width of the RI since it might indicate insufficient sample numbers.

All calculations and plotting conducted throughout this study were performed using SAS version 9.4 (SAS Institute Inc) and Microsoft Office Excel software (Microsoft Corp.).

2.9 | Ethical approval

Applicable national, international, and institutional guidelines for studies involving live animals were followed. The Finnish national Animal Experiment Board, permit number: ESAVI/7482/04.10.07/2015 (date of approval 9.10.2015) approved this study. For samples purposefully collected for use in the NMR metabolomics project, written informed consent from animal owners was obtained, allowing the scientific use of samples and metadata. For the leftover laboratory samples, a consent for leftover sample material used for scientific purposes was given during analysis ordering.

3 | RESULTS

3.1 | Proton NMR spectroscopy quantitates 123 measurands

The NMR platform was able to quantify 123 measurands, including extensive lipoprotein profiling, fatty acids, amino acids, albumin, creatinine, and glycolysis-related metabolites, in canine serum/plasma

FIGURE 1 The measurands quantified by the nuclear magnetic resonance-based canine metabolomics platform. *not available from EDTA plasma samples. BCAA, Branched-chain amino acids; L-HDL, large HDL particles; L-LDL, large LDL particles; L-VLDL, large VLDL particles; S-HDL, small HDL particles; S-LDL, small LDL particles; S-VLDL, small VLDL particles; XL-HDL, very large HDL particles; XL-VLDL, Chylomicrons and very large VLDL particles

MEASURANDS

Creatinine mmol/l

Albumin g/l

Glycolysis related metabolites mmol/l

Glucose
Lactate
Pyruvate*
Citrate
Acetate

Triglycerides mmol/l

Triglycerides
VLDL triglycerides
LDL triglycerides
HDL triglycerides

Inflammation mmol/l

Glycoprotein acetyls

Amino acids

mmol/l
Alanine
Glutamine
Glycine*
Histidine
Isoleucine
Leucine
Valine
Phenylalanine
Tyrosine
BCAA

Ratio
Glycine/BCAA
BCAA/Tyrosine
Phenylalanine/tyrosine
Glycine/valine
Alanine/BCAA
Alanine/valine

Cholesterol mmol/l

Cholesterol
VLDL cholesterol
LDL cholesterol
HDL cholesterol
Free cholesterol
Esterified cholesterol

Lipoprotein particles

mmol/l
VLDL particles
LDL particles
HDL particles
VLDL lipids
LDL lipids
HDL lipids

nm
VLDL particle size
LDL particle size
HDL particle size

Fatty acids

mmol/l
Palmitic acid
Stearic acid
Oleic acid
Linoleic acid
Arachidonic acid
Docosapentaenoic acid
Docosahexaenoic acid
Omega-3 fatty acids
Omega-6 fatty acids
Polyunsaturated fatty acids
Saturated fatty acids
Total fatty acids

% of total fatty acids
Palmitic acid%
Stearic acid%
Oleic acid%
Linoleic acid%
Arachidonic acid%
Docosapentaenoic acid%
Docosahexaenoic acid%
Omega-3 fatty acids%
Omega-6 fatty acids%
Polyunsaturated fatty acids%
Saturated fatty acids%

Ratio
Omega-6/omega-3 fatty acids

Lipoprotein subclass particle concentration and composition mmol/l

XL-VLDL particles
L-VLDL particles
S-VLDL particles
L-LDL particles
S-LDL particles
XL-HDL particles
L-HDL particles
S-HDL particles
XL-VLDL lipids
L-VLDL lipids
S-VLDL lipids
L-LDL lipids
S-LDL lipids
XL-HDL lipids
L-HDL lipids
S-HDL lipids
XL-VLDL cholesterol
L-VLDL cholesterol
S-VLDL cholesterol
L-LDL cholesterol
S-LDL cholesterol
XL-HDL cholesterol
L-HDL cholesterol
S-HDL cholesterol
XL-VLDL esterified cholesterol
L-VLDL esterified cholesterol
S-VLDL esterified cholesterol
L-LDL esterified cholesterol
S-LDL esterified cholesterol
XL-HDL esterified cholesterol
L-HDL esterified cholesterol
S-HDL esterified cholesterol
XL-VLDL free cholesterol
L-VLDL free cholesterol
S-VLDL free cholesterol
L-LDL free cholesterol
S-LDL free cholesterol
XL-HDL free cholesterol
L-HDL free cholesterol
S-HDL free cholesterol
XL-VLDL triglycerides
L-VLDL triglycerides
S-VLDL triglycerides
L-LDL triglycerides
S-LDL triglycerides
XL-HDL triglycerides
L-HDL triglycerides
S-HDL triglycerides
XL-VLDL phospholipids
L-VLDL phospholipids
S-VLDL phospholipids
L-LDL phospholipids
S-LDL phospholipids
XL-HDL phospholipids
L-HDL phospholipids
S-HDL phospholipids

(Figure 1). The sample failure rate was around 0.4%, with the most common cause being that the sample volume was too small. The per-metabolite failure rate was typically under 1%. However, measurands having very low concentrations in healthy dogs, such as chylomicrons and very large VLDL (XL-VLDL), large VLDL (L-VLDL), and HDL triglyceride variables, had 3%-43% missing observations.

3.2 | Precision of the platform

We studied analytic precision using three biologically different dogs, with duplicate aliquots of each dog's sample analyzed once a day during a 20-day period (Table 2; Table S3). The aliquots from a senior dog suffering from hyperadrenocorticism showed marked chylomicronemia and were thus excluded from method precision estimations. Outlier removal affected the interpretation of results slightly; thus, results were evaluated with outliers removed. In the main (nonchylomicronemic) samples evaluated, 103/123 measurands reached all precision goals. Albumin, glucose, and creatinine did not reach the precision goal, CV_{BV}, based on biological variation, but reached all other precision goals, including the CV% goal based on ASVCP Clinical Decision Limits (CV_{CDL}), and the S_T goal of being under 1/8 reference widths (S_{Tmax}). Histidine, acetate, phenylalanine/tyrosine, and branched-chain amino acids (BCAA)/tyrosine met the precision goal of CV% under 20% (CV_{BG}) but did not meet the S_{Tmax} goal. Ten of 123 measurands did not reach the CV_{BG} goal but reached the S_{Tmax} goal. All of these measurands from the tested samples were lipid measurands with high inter-individual biological variation, large RI widths, and were in low concentrations. The CV_{BG} goal was thus regarded as inappropriate for these measurands since the variation of these measurand concentrations was not considered to affect clinical decision-making.

Chylomicronemia caused imprecision in multiple lipid measurands, creatinine, leucine, and phenylalanine. Glutamine precision was nonevaluable since the olefin oligomer gel in the sample tubes inhibited glutamine quantification. The precision of XL-VLDL phospholipids was nonevaluable due to multiple missing observations caused by very low measurand concentrations physiologically. The concentration of XL-VLDL particles was too low in the nonlipemic dogs for reliable precision estimations.

3.3 | Interference screen

We studied the interference of hemolysis, lipemia, and unconjugated and conjugated bilirubin using triplicate samples of two different hemolysis and bilirubinemia test pools and one lipemia test pool (Table S4; Table 3). Hemolysis interfered with the quantitation of albumin and certain lipids. No interference was observed for unconjugated bilirubin. The only measurands exceeding the interference cutoff for conjugated bilirubin were L-VLDL cholesterol, L-VLDL esterified cholesterol, and S-HDL triglycerides. However, multiple missing observations were present, and thus, interference could not be inferred. Lipemia affected the quantitation of GlycA due to the contribution of lipid to the GlycA signal. However, the concentration of GlycA remained above the reference interval after the removal of lipids, indicating that lipemia was not the sole cause for the increased GlycA concentration in the lipemia pool.

3.4 | Method comparison

Using 999 canine clinical samples, we evaluated whether the results of routine clinical chemistry measurands using the NMR metabolomics platform were comparable with the results obtained using conventional clinical chemistry methods (Table 4; Table S5). The tested measurands were glucose, lactate, creatinine, albumin, cholesterol, and triglycerides. The tested samples covered the clinical concentration ranges for all measurands. The comparison plots showed satisfactory agreement between the two methods for all measurands. In some measurands, logarithm transformation showed better agreement than the initial data.

Glucose, lactate, and albumin measurements reached the TE_a goals based on ASVCP Clinical Decision Limits,[25] indicating that the two methods can be used interchangeably without affecting clinical decision-making (Table 4). The bias percentage of creatinine, cholesterol, and triglycerides was too high for the measurands to reach the TE_a goals based on Clinical Decision Limits, meaning that the two methods could not be used interchangeably. Values obtained by the conventional method could be evaluated against values obtained by the NMR method using the slope and intercept calculated with Deming regression (Table S5).

3.5 | The effect of sample storage on measurand stability

All evaluated measurands remained stable in refrigerated temperatures for 7 days (Table 5, Table S1). Most of the measurands also remained stable at room temperature for 7 days. Unstable measurands at room temperature were the histidine, isoleucine, glutamine, tyrosine, and phenylalanine amino acids, acetate, triglycerides, certain lipoprotein particle constituent concentrations, as well as the fatty acid, docosapentaenoic acid. The stability of all amino acids other than glutamine was better in EDTA plasma than in serum. Glutamine concentrations changed significantly at 4 days of storage at room temperature in EDTA plasma samples and 7 days of storage in serum samples. The acceptable change limit (ACL) of glutamine and XL-VLDL phospholipids could not be calculated using the mean coefficient of variation (CVa) analysis since these were not available from the precision study. The changes observed in triglyceride concentrations at room temperature originated from their computational quantitation based on the lipoprotein particle distribution and lipid quantity in the lipoprotein particle core and outer surface. These are slightly affected by sample storage at room temperature, thus affecting triglyceride quantitation.

All of the evaluated measurands were stable at −80°C for 12 months. Storage at −20°C affected certain lipoprotein particles and composition, certain fatty acids, citrate, acetate, and GlycA.

TABLE 2 Measurands exceeding one or more of their within-laboratory precision goals using a nuclear magnetic resonance platform in dogs

				Precision		Precision goals			
Measurand	Dog	N_{miss}	Mean	CV%	S_T	CV_{BV} (%)	CV_{CDL} (%)	CV_{BG} (%)	S_{Tmax}
Glucose mmol/L	adu	0	4.51	5.0	0.23	3.9[a]	10.0		0.30
Creatinine μmol/L	pup	0	61.8	6.2	3.9	5.9[a]	10.0		9.0
Creatinine μmol/L	adu	0	54.8	7.5	4.1	5.9[a]	10.0		9.0
Albumin g/L	pup	0	28.8	2.1	0.6	1.0[a]	7.5		0.9
Albumin g/L	adu	0	29.8	2.1	0.6	1.0[a]	7.5		0.9
Acetate μmol/L	pup	0	33.1	7.4	2.5			20	2.0[a]
Acetate μmol/L	adu	0	31.0	12.3	3.8			20	2.0[a]
Histidine μmol/L	pup	0	73.4	9.9	7.3			20	5.6[a]
BCAA/Tyrosine	adu	0	8.0	10.2	0.8			20	0.7[a]
Phenylalanine/Tyrosine	adu	0	0.8	10.2	0.1			20	0.1[a]
HDL triglycerides mmol/L	pup	0	0.023	22.3	0.005			20[a]	0.009
XL-VLDL lipids mmol/L	pup	1	0.024	39.7	0.010			20[a]	0.033
XL-VLDL lipids mmol/L	adu	0	0.016	51.2	0.008			20[a]	0.033
XL-VLDL cholesterol mmol/L	pup	1	0.001	37.9	0.000			20[a]	0.007
XL-VLDL cholesterol mmol/L	adu	0	0.007	24.9	0.002			20[a]	0.007
XL-VLDL free cholesterol mmol/L	adu	1	0.003	30.7	0.001			20[a]	0.006
XL-VLDL triglycerides mmol/L	pup	1	0.023	40.5	0.009			20[a]	0.021
L-VLDL triglycerides mmol/L	adu	0	0.108	20.0	0.022			20[a]	0.052
XL-HDL triglycerides mmol/L	pup	0	0.009	20.5	0.002			20[a]	0.003
XL-HDL triglycerides mmol/L	adu	1	0.010	20.1	0.002			20[a]	0.003
L-HDL triglycerides mmol/L	pup	0	0.012	22.2	0.003			20[a]	0.003
S-HDL triglycerides mmol/L	adu	4	0.004	49.5	0.002			20[a]	0.004
L-VLDL phospholipids mmol/L	pup	2	0.009	60.8	0.005			20[a]	0.013
L-VLDL phospholipids mmol/L	adu	0	0.030	27.9	0.008			20[a]	0.013

Note: The method's within-laboratory precision, expressed as coefficient of variation (CV%) and standard deviation (S_T), was evaluated against precision goals CV_{BV}, CV_{CDL}, CV_{BG}, and S_{Tmax}. CV_{BV} represents the biological variation-based goal for CV%.[24] CV_{CDL} represents the goal for CV% based on total allowable error.[25] CV_{BG} was used as the CV% goal for measurands, for which CV_{BV} and CV_{CDL} goals were unavailable and represent the generally accepted laboratory error of 20% in metabolomics. ST_{max} represents the goal for ST, set as 1/8 of the reference interval width. Precision was evaluated from the aliquots (n = 40) of the two dogs, puppy (pup) and adult (adu), with nonlipemic serum. Only lipid measurands with large reference interval width, and low concentrations in the tested samples, exceeded the CV_{BG} goal. The CV_{BG} goal was regarded inappropriate for these measurands in these conditions since the detected imprecision was not considered to affect clinical decision-making.

Abbreviations: L-HDL, large HDL particles; L-VLDL, large VLDL particles; S-HDL, small HDL particles; XL-HDL, very large HDL particles; XL-VLDL, Chylomicrons and very large VLDL particles.

N_{miss}, number of missing observations; Mean, overall mean of the dog's measured aliquots' measurand concentrations.

[a]Precision goal exceeded.

TABLE 3 Measurands with detected interference

Interferent	Measurand	Dog	$Mean_{base}$	$Mean_{test}$	d_c	d_{obs}	d_{obs_lower}
Lipemia	Glycoprotein acetyls μmol/L		1000	1267	16	266	217
Hemolysis	Albumin g/L	1	27.4	31.5	0.6	4.1	2.4
Hemolysis	Albumin g/L	2	28.5	32.5	0.6	4.0	2.3
Hemolysis	Triglycerides mmol/L	2	0.612	0.384	0.051	0.228	0.070
Hemolysis	VLDL particles nmol/L	1	40	27	2	12	6
Hemolysis	VLDL particle size nm	1	40.15	42.52	0.35	2.37	1.28
Hemolysis	LDL particle size nm	1	22.30	21.91	0.04	0.39	0.27
Hemolysis	VLDL cholesterol mmol/L	1	0.197	0.131	0.012	0.066	0.030
Hemolysis	LDL triglycerides mmol/L	1	0.235	0.163	0.016	0.072	0.023
Hemolysis	LDL triglycerides mmol/L	2	0.216	0.130	0.016	0.086	0.037
Hemolysis	Docosahexaenoic acid mmol/L	2	0.236	0.162	0.014	0.074	0.029
Hemolysis	Omega-3 fatty acids mmol/L	2	0.772	0.575	0.043	0.197	0.064

Note: Interference was determined to be present, when the point estimate (d_{obs}) exceeded the lower 95% confidence interval limit of the interference cutoff value (d_{obs_lower}). The table only includes lipoprotein particle superclass results, lipoprotein subclass results are not included. N = 3 for all samples. Interference for conjugated and unconjugated bilirubin was also screened. No interference was detected for unconjugated bilirubin. The only measurands exceeding the interference cutoff for conjugated bilirubin were L-VLDL cholesterol, L-VLDL esterified cholesterol, and S-HDL triglycerides. However, multiple observations were missing for these measurands, and thus, interference could not be inferred.

d_c, interference cutoff value; $Mean_{base}$, mean of the measurand results of the control replicates in the respective analyte units; $Mean_{test}$, mean of the results of the replicate test samples in the respective analyte units.

TABLE 4 Method interchangeability according to the method comparison study

Measurand	Bias (%)	CVa (%)	TE_{obs} (%)	TE_a
Glucose	3.8	3.7	11	20
Lactate	4.6	3.2	11	40
Creatinine	15.7	6.9	29*	20
Albumin	3.1	2.1	7	15
Cholesterol	23.4	2.9	29*	20
Triglycerides	29.3	8.6	46*	25

Note: Nuclear magnetic resonance (NMR) results of the clinical chemistry measurands included on the NMR platform compared with conventional clinical chemistry analysis methods. Bias was only evaluated as a measure for method interchangeability since results obtained by the conventional methods cannot be viewed as true values of the measurands. The results were determined to be interchangeable, if the observed total error (TE_{obs}) was below the total allowable error (TE_a) based on clinical decision limits.[25]

CVa, Mean CV% of the two nonlipemic dogs from the precision study.

Citrate concentration was significantly ($P < .05$) changed at 1 week of −20°C storage.

Stored heparinized plasma samples rarely reached a *P*-value of .05, even when the MPD exceeded the ACL. Statistically significant ($P < .05$) changes were also noted in other sample types.

Outlier removal affected the interpretation of serum and EDTA plasma results slightly; thus, those results were evaluated after outlier removal. In heparinized plasma, outliers could not be reliably identified due to the small sample size (n = 4).

3.6 | The effect of delayed plasma separation on measurand stability

We studied the effect of delayed plasma separation in 34 EDTA plasma samples, which had been stored as whole blood in the refrigerator for 24 or 48 hours before separating plasma (Table S6; Table 6). Outlier removal affected the interpretation of results slightly; thus, the results were evaluated after outlier removal. Prolonged contact with red blood cells (RBCs) affected the concentration of many measurands. Significant changes ($P < .05$, MPD > ACL) after a 1-day contact with RBCs at refrigerated temperatures were observed for glucose and lactate. After 2 days of contact with RBCs, significant changes were observed in citrate, the amino acids, alanine, histidine, isoleucine, leucine, valine, phenylalanine, and tyrosine, and cholesterol, triglycerides, and lipoprotein particles and their constituents. Glutamine values also changed significantly ($P < .05$) at 48 hours of storage in whole blood, but the MPD could not be evaluated against the ACL due to the missing ACL.

3.7 | Sample tube validation

We studied the differences of seven different sample tubes and the variability between two sample lots using samples from 20 client-owned dogs (Table S2). Outlier removal did not affect the interpretation of results; thus, results were evaluated without outlier removal.

First, the different sample tubes were compared with the primary reference tube (Vacuette Lithium Heparin). Most measurands showed comparable results for all tube types. Significant differences ($P < .05$)

TABLE 5 Critical storage times for measurands affected by certain storage conditions in at least one sample type

Measurand	Temperature	Storage time$_{Serum}$ (p)	Storage time$_{EDTA}$ (p)	Storage time$_{HP}$ (p)
Glutamine	RT	7 d[a] (0.00*)	4 d[a] (0.04*)	4 d[a] (0.11)
Histidine	RT	3 d (0.00*)	NO	3 d (0.03*)
Isoleucine	RT	2 d (0.04*)	NO	4 d (0.20)
Phenylalanine	RT	3 d (0.00*)	NO	7 d (0.06)
Tyrosine	RT	3 d (0.01*)	NO	NO
HDL triglycerides	RT	2 d (0.01*)	4 d (0.07)	4 d (0.20)
LDL triglycerides	RT	2 d (0.01*)	4 d (0.02*)	2 d (0.20)
VLDL triglycerides	RT	2 d (0.00*)	2 d (0.04*)	4 d (0.11)
Triglycerides	RT	24 h (0.04*)	2 d (0.02*)	2 d (0.20)
Acetate	RT	4 d (0.02*)	7 d (0.00*)	4 d (0.03*)
Docosapentaenoic acid	RT	7 d (0.03*)	NE	7 d (0.20)
Citrate	−20°C	1 wk (0.00*)	NO	—
Acetate	−20°C	3 mo (0.00*)[b]	6 mo (0.00*)	—
Glycoprotein acetyls	−20°C	1 mo (0.00*)	1 month (0.01*)	—
Omega-3 fatty acids	−20°C	6 mo (0.02*)	NE	—
HDL particle size	−20°C	3 mo (0.04*)	3 mo (0.03*)	—
HDL particles	−20°C	6 mo (0.00*)	6 mo (0.00*)	—
LDL lipids	−20°C	NE	6 mo (0.03*)	—
LDL particles	−20°C	NE	6 mo (0.03*)	—
LDL cholesterol	−20°C	NE	6 mo (0.03*)	—
VLDL particles	−20°C	6 mo (0.04*)	6 mo (0.01*)	—

Note: The storage time presented in the table represents the time point, where statistically significant changes (two-sided Wilcoxon exact test $P < .05$), with a mean percentage deviation (MPD) exceeding the acceptable change limit (ACL) are first observed. In sample types where statistical significance was not observed ($P \geq .05$), the storage times represent the time point, where the MPD first exceeds the ACL. This table includes only primary measurands. Lipoprotein particle subclasses are not included. Stability at −20 and −80°C was not studied in heparinized plasma. $n_{serum} = 7$, $n_{EDTA} = 7$, $n_{HP} = 4$.

NE, not evaluable due to discrepancies in *P*-values and MPD vs ACL. Results in other sample types should be consulted until further studies are conducted.

Abbreviations: NO, no change observed; RT, room temperature.

[a] ACL unknown, evaluated only as $P < .05$, time in heparinized plasma represents the time, where a change in magnitude similar to that in serum and EDTA plasma is observed.

[b] At 6 mo of storage, MPD returned below the ACL and $P > .05$.

*$P < .05$.

with MPD > ACL were noted for citrate, glucose, lactate, and GlycA, and significant differences ($P < .05$) with MPD < ACL were observed for glutamine, histidine, pyruvate, and acetate. Glutamine showed a significant difference ($P < .05$), but its ACL was not available. Most of the variability between tubes was observed between serum and plasma.

Second, measurand values in all serum tubes were compared with the reference serum tube (Vacuette Z Serum Clot Activator). All serum tubes showed comparable results with the serum reference tube.

The only lot-to-lot variability was observed in MiniCollect Serum gel tubes for glutamine and GlycA. Results obtained with both of these tube lots remained comparable to the reference serum tube (Vacuette Z Serum Clot Activator). In the MiniCollect Serum gel tubes, the two lots represented tubes of the old-type (lot A) and new-type (lot B) tubes; the physical appearance of the tube changed, but the constituents of the tube remained the same.

3.8 | Reference intervals for puppies, adult, and senior dogs

We determined the RIs of the nuclear magnetic resonance method for 123 measurands in serum and heparinized plasma samples (Table 7). Raw, unrounded RIs and their 90% CI are presented in Table S7.

In certain lipoprotein measurands, such as the XL-VLDL variables, the concentration in healthy animals was very low, causing highly skewed distributions. Also, automatic quality control rejection of extremely low values caused an inability to calculate CIs with the nonparametric method used. For these measurands, the lower reference limit was rounded to 0. For a multitude of measurands, the 90% CI width was > 20% of the RI width due to skewed or heavy-tailed distributions. This was especially observed in RIs for puppies and senior dogs, where the n count was lower (n < 170) than in the adult

TABLE 6 Critical storage times as whole blood for measurands affected by storage as whole blood

Measurand	Storage time (p)
Glucose	24 h (0.00)
Lactate	24 h (0.00)
Citrate	48 h (0.00)
Alanine	48 h (0.00)
Glutamine	48 h[a] (0.00)
Histidine	48 h (0.00)
Isoleucine	48 h (0.00)
Leucine	48 h (0.01)
Valine	48 h (0.00)
Phenylalanine	48 h (0.00)
Tyrosine	48 h (0.00)
HDL lipids	48 h (0.03)
HDL particles	48 h (0.00)
LDL diameter	48 h (0.00)
VLDL diameter	48 h (0.04)
Cholesterol	48 h (0.02)
HDL cholesterol	48 h (0.00)
Esterified cholesterol	48 h (0.00)
Triglycerides	48 h (0.00)
VLDL triglycerides	48 h (0.00)

Note: The storage time presented in the table represents the time point, where statistically significant changes (two-sided Wilcoxon exact test $P < .05$) together with a mean percentage deviation (MPD) exceeding the acceptable change limit (ACL) are first observed. n = 34. This table includes only primary measurands. Lipoprotein particle subclasses are not included.

[a]ACL unknown.

dogs. However, we also saw this phenomenom in RIs with very high n counts (n > 800).

4 | DISCUSSION

Metabolomics is a rapidly growing field, with considerable potential for numerous clinical and scientific applications. NMR spectroscopy is a promising metabolomics method for clinical use due to its quantitative nature, high throughput, accuracy, and speed.[16] In this study, we validated a novel, cost-effective NMR metabolomics platform in dogs. A similar approach has been demonstrated previously and widely used in people.[16] The platform quantifies 123 measurands from 100 μL samples of serum, heparinized plasma, EDTA plasma, or citrated plasma. The throughput for one device is ~200 samples per 24 hours, making around 70 000 samples per year. The turnaround time is currently 5 days and can be reduced. These characteristics make it a potentially new high-throughput method to facilitate veterinary research and clinical diagnostics, with significant implications regarding the treatment, care, and well-being of dogs.

Using a new laboratory method in clinics requires excellent methodologic precision. The precision of the NMR metabolomics platform is generally outstanding, as demonstrated by our results, showing that most of the measurands reached all respective precision goals. However, for histidine, acetate, phenylalanine/tyrosine, and BCAA/tyrosine, further studies are needed in diseased animals to conclude whether measurement imprecision affects clinical use.

The analytical method used should always be considered when evaluating laboratory results against treatment guidelines and RIs.[14] A linear relationship between the conventional and NMR method was observed for all evaluated clinical chemistry measurands: glucose, lactate, creatinine, albumin, triglycerides, and cholesterol. However, the results of these measurands cannot be evaluated interchangeably between methods. Triglycerides showed the highest between-method variability. This can be explained by differences in methodology (NMR measures triglycerides within lipoprotein particles vs the conventional method interacts solely with the triglyceride molecule), sample handling (immediate analysis with the conventional method vs freezing of NMR aliquots, one sample batch partly thawed during shipment), and biological characteristics (effect of lipemia on precision, causing higher variability in high triglyceride concentrations).

Correct preanalytical measures are considered essential for the integrity of laboratory results.[39,40] For instance, an appropriate sample drawing technique is crucial to avoid hemolysis, fasting before sample drawing is critical to avoid lipemia, and prompt RBC separation is needed to avoid prolonged RBC metabolism, which all impact NMR analytic results. The profound impact of chylomicronemia on method precision was caused by matrix heterogeneity due to chylomicron cream layer formation, which could be reduced by sample mixing. In addition to glycolysis-related metabolites, amino acids were affected by prolonged contact with RBC. This might explain why amino acid stability was better in our storage study using plasma and serum samples than in previous studies using whole blood.[41,42]

It is critical to use sample storage and shipping conditions that allow for sample quality preservation. All tested measurands remained stable at refrigerator temperature for 1 week, making it the optimal short-term storage temperature for samples. Storage at room temperature should be avoided. The stability of most amino acids was better in EDTA plasma than in serum at room temperature, which has been previously reported.[42] The gold standard for long-term storage is immediate freezing to −80°C after serum/plasma separation and avoiding additional freeze-thaw cycles.[43] This was also the optimal storage protocol according to this study, with all measurands remaining stable for 1 year at −80°C. Two weeks of storage at −20°C was suitable for most measurands.

Determination of RIs is an important prerequisite for the clinical use of a new laboratory method. We determined the RIs for 123 measurands, most of which have not been previously published. The sample tube validation study suggested that the sample tubes of

TABLE 7 Measurand reference intervals determined on a canine nuclear magnetic resonance-based metabolomics platform

Measurand	HP all dogs	S all dogs	S puppy	S adult	S senior
Glucose mmol/L	4.3-6.8	4.4-6.8	4.7-7.3	4.4-6.6	4.0-6.1
Lactate mmol/L	0.7-3.0	1.1-3.6	1.3-2.9	1.1-3.6	1.1-4.2
Creatinine µmol/L	40-99	32-103	21-96	40-108	37-104
Albumin mg/dL	26-32	25-32	22-31	26-32	26-32
Cholesterol mmol/L	3.8-10.4	3.6-10.3	3.8-10.1	3.6-10.3	3.5-10.6
Triglycerides mmol/L	0.22-0.97	0.19-1.00	0.18-0.76	0.19-0.97	0.19-1.13
Pyruvate µmol/L	29-155	11-107	9-96	11-111	13-109
Citrate µmol/L	63-122	61-123	57-115	61-124	62-128
Acetate µmol/L	19-36	21-37	22-40	21-37	20-37
Alanine µmol/L	214-584	216-597	205-504	205-583	244-650
Glycine µmol/L	147-466	130-454	145-594.	127-381	128-403
Glutamine µmol/L	570-919	640-1015	642-997	659-1028	616-1014
Histidine µmol/L	50-91	53-98	46-93	55-99	55-97
Isoleucine µmol/L	33-80	37-89	30-80	38-92	38-87
Leucine µmol/L	74-168	83-185	67-159	89-186	94-186
Valine µmol/L	107-245	113-251	80-236	119-260	124-253
Phenylalanine µmol/L	32-64	30-65	28-66	29-64	34-66
Tyrosine µmol/L	39-85	41-89	39-83	41-90	46-93
BCAA µmol/L	222-482	242-515	178-462	261-524	251-521
Glycine/BCAA	0.3-1.4	0.3-1.5	0.4-3.0	0.3-1.2	0.3-1.2
BCAA/Tyrosine	3.7-9.5	3.8-9.2	3.8-8.3	3.9-9.5	3.5-8.6
Phenylalanine/tyrosine	0.6-1.2	0.5-1.0	0.5-1.1	0.5-1.0	0.5-1.0
Glycine/valine	0.6-2.6	0.7-3.0	0.9-6.8	0.7-2.5	0.7-2.6
Alanine/BCAA	0.7-2.0	0.6-1.6	0.6-1.4	0.5-1.6	0.6-1.8
Alanine/valine	1.3-3.7	1.2-3.5	1.3-3.0	1.1-3.5	1.3-3.8
VLDL lipids mmol/L	0.1-1.1	0.1-1.2	0.1-0.8	0.1-1.1	0.1-1.3
VLDL particles nmol/L	15-54	12-54	14-45	12-51	12-67
LDL lipids mmol/L	0.5-3.7	0.7-3.7	0.7-3.7	0.7-3.6	0.7-4.5
LDL particles nmol/L	200-1400	240-1300	270-1300	240-1300	240-1600
HDL lipids mmol/L	7.7-14.6	6.9-15.1	7.2-15.0	6.9-15.1	6.6-15.3
HDL particles nmol/L	32 000-57 000	30 000-58 000	33 000-58 000	30 000-59 000	28 000-58 000
VLDL particle size nm	35.8-43.6	35.2-43.8	35.1-41.7	35.3-43.8	35.4-44.4
LDL particle size nm	22.1-23.9	22.2-23.5	22.3-23.5	22.2-23.4	22.2-23.5
HDL particle size nm	10.1-10.7	10.1-10.7	10.1-10.6	10.0-10.6	10.1-10.7
VLDL cholesterol mmol/L	0.03-0.31	0.03-0.31	0.03-0.23	0.03-0.29	0.03-0.36
LDL cholesterol mmol/L	0.16-2.35	0.28-2.27	0.30-2.24	0.28-2.21	0.28-2.85
HDL cholesterol mmol/L	3.6-8.0	3.2-7.9	3.6-7.9	3.2-7.9	3.0-8.0
Esterified cholesterol mmol/L	3.1-8.2	2.9-8.1	3.2-8.0	2.9-8.1	2.9-8.4
Free cholesterol mmol/L	0.7-2.2	0.6-2.2	0.7-2.0	0.6-2.2	0.6-2.4
VLDL triglycerides mmol/L	0.02-0.70	0.00-0.70	0.00-0.44	0.00-0.69	0.00-0.86
LDL triglycerides mmol/L	0.13-0.30	0.13-0.31	0.15-0.33	0.13-0.29	0.14-0.27
HDL triglycerides mmol/L	0.01-0.08	0.00-0.08	0.00-0.04	0.00-0.07	0.01-0.09
Glycoprotein acetyls µmol/L	532-964	597-1028	611-996	596-1005	605-1129
Palmitic acid mmol/L	1.9-3.5	1.8-3.6	1.8-3.4	1.8-3.6	1.8-3.9
Stearic acid mmol/L	1.9-3.8	1.7-3.8	1.8-3.7	1.7-3.9	1.7-4.0

(Continues)

TABLE 7 (Continued)

Measurand	HP all dogs	S all dogs	S puppy	S adult	S senior
Oleic acid mmol/L	1.1-2.5	1.3-2.8	1.3-2.5	1.3-2.8	1.3-3.2
Linoleic acid mmol/L	2.7-5.7	2.5-5.9	2.5-5.1	2.5-5.9	2.5-6.2
Arachidonic acid mmol/L	1.6-3.7	1.4-3.6	1.5-3.7	1.4-3.6	1.3-3.7
Docosapentaenoic acid mmol/L	0.1-0.3	0.1-0.4	0.1-0.3	0.1-0.4	0.1-0.4
Docosahexaenoic acid mmol/L	0.1-0.9	0.1-0.7	0.1-0.8	0.1-0.7	0.1-0.8
Omega-3 fatty acids mmol/L	0.4-1.8	0.4-1.6	0.4-1.6	0.4-1.6	0.5-1.8
Omega-6 fatty acids mmol/L	4.5-9.6	4.1-9.8	4.2-9.3	4.1-9.6	3.9-10.6
Polyunsaturated fatty acids mmol/L	5.2-10.8	4.7-11.1	4.8-10.6	4.7-11.0	4.6-12.0
Saturated fatty acids mmol/L	3.9-7.2	3.6-7.4	3.6-6.8	3.6-7.5	3.5-7.7
Total fatty acids mmol/L	10.5-20.4	9.7-21.0	9.8-20.0	9.7-21.2	9.3-22.5
Palmitic acid %	15.9-19.8	16-20	16-19	16-19	16-20
Stearic acid %	17.5-19.5	17.3-19.4	17.5-19.3	17.4-19.5	17.1-19.4
Oleic acid %	9.2-14.2	10.7-15.1	10.6-14.2	10.9-15.1	10.9-15.7
Linoleic acid %	23.9-29.4	24.1-28.6	23.3-27.5.	24.5-28.6	24.7-28.7
Arachidonic acid %	13.6-20.7	13.2-20.1	15.0-20.7.	13.1-19.9	12.7-19.2
Docosapentaenoic acid %	0.9-1.9	1.1-2.0	1.1-1.9	1.1-1.9	1.1-2.0
Docosahexaenoic acid %	0.9-6.2	0.7-4.8	1.0-5.2	0.7-4.7	0.6-5.0
Omega-3 fatty acids %	3-12	3-10	3-11	3-11	4-11
Omega-6 fatty acids %	43-49	42-48	42-48	42-48	42-47
Polyunsaturated fatty acids %	48-57	48-55	48-55	47-55	47-55
Saturated fatty acids %	34-39	34-38	34-38	34-38	33-38
Omega-6/omega-3 fatty acids	3.5-16.3	4.2-13.4	4.1-13.5	4.2-14.2	4.0-13.1
XL-VLDL particles nmol/L	0-2	0-1	0-1	0-1	0-2
L-VLDL particles nmol/L	1-15	0-16	0-11	0-15	0-19
S-VLDL particles nmol/L	11-38	10-39	13-34	10-37	10-47
L-LDL particles nmol/L	79-380	90-370	110-350	91-380	83-400
S-LDL particles nmol/L	89-1000	140-960	130-940	130-920	140-1100
XL-HDL particles nmol/L	1-6200	0-5800	25-5500	0-5800	1-6000
L-HDL particles nmol/L	21 000-34 000	20 000-34 000	20 000-34 000	19 000-35 000	18 000-34 000
S-HDL particles nmol/L	11 000- 19 000	11 000-20 000	11 000-19 000	11 000-20 000	10 000-20 000
XL-VLDL lipids mmol/L	0-0.3	0-0.3	0-0.2	0-0.2	0-0.4
L-VLDL lipids mmol/L	0-0.6	0-0.6	0-0.4	0-0.6	0-0.7
S-VLDL lipids mmol/L	0.1-0.4	0.1-0.4	0.1-0.3	0.1-0.3	0.1-0.4
L-LDL lipids mmol/L	0.2-1.2	0.3-1.2	0.3-1.1	0.3-1.2	0.3-1.3
S-LDL lipids mmol/L	0.2-2.6	0.3-2.4	0.3-2.4	0.3-2.3	0.3-2.9
XL-HDL lipids mmol/L	0.6-5.2	0.6-5.0	0.6-4.7	0.6-5.0	0.7-5.2
L-HDL lipids mmol/L	5.4-8.1	5.0-8.3	5.0-8.1	4.9-8.3	4.8-8.3
S-HDL lipids mmol/L	1.3-2.1	1.3-2.2	1.3-2.1	1.3-2.2	1.1-2.2
XL-VLDL cholesterol mmol/L	0-0.06	0-0.06	0-0.04	0-0.06	0-0.08
L-VLDL cholesterol mmol/L	0-0.13	0-0.13	0-0.08	0-0.12	0-0.15
S-VLDL cholesterol mmol/L	0.01-0.14	0.02-0.15	0.02-0.11	0.02-0.14	0.02-0.18
L-LDL cholesterol mmol/L	0.03-0.70	0.07-0.68	0.11-0.61	0.07-0.68	0.07-0.76
S-LDL cholesterol mmol/L	0.09-1.64	0.16-1.53	0.16-1.50	0.16-1.48	0.18-1.88
XL-HDL cholesterol mmol/L	0.3-3.0	0.2-2.8	0.3-2.9	0.2-2.7	0.2-2.9
L-HDL cholesterol mmol/L	2.6-4.3	2.3-4.4	2.5-4.3	2.3-4.5	2.2-4.4

(Continues)

TABLE 7 (Continued)

Measurand	HP all dogs	S all dogs	S puppy	S adult	S senior
S-HDL cholesterol mmol/L	0.5-1.0	0.5-1.0	0.6-1.0	0.5-1.0	0.5-1.0
XL-VLDL esterified cholesterol mmol/L	0-0.03	0-0.03	0-0.02	0-0.03	0-0.03
L-VLDL esterified cholesterol mmol/L	0-0.05	0-0.06	0-0.04	0-0.05	0-0.07
S-VLDL esterified cholesterol mmol/L	0-0.09	0.01-0.09	0.01-0.08	0.01-0.09	0.01-0.11
L-LDL esterified cholesterol mmol/L	0.01-0.51	0.03-0.49	0.05-0.43	0.02-0.48	0.02-0.55
S-LDL esterified cholesterol mmol/L	0.05-1.14	0.10-1.09	0.10-1.06	0.10-1.06	0.12-1.35
XL-HDL esterified cholesterol mmol/L	0.2-2.3	0.2-2.1	0.2-2.1	0.2-2.1	0.2-2.3
L-HDL esterified cholesterol mmol/L	2.3-3.7	2.0-3.8	2.2-3.7	2.0-3.8	1.9-3.8
S-HDL esterified cholesterol mmol/L	0.4-0.8	0.4-0.8	0.5-0.8	0.4-0.8	0.4-0.8
XL-VLDL free cholesterol mmol/L	0-0.05	0-0.05	0-0.03	0-0.04	0-0.05
L-VLDL free cholesterol mmol/L	0-0.08	0-0.08	0-0.05	0-0.07	0-0.10
S-VLDL free cholesterol mmol/L	0.01-0.05	0.01-0.06	0.01-0.05	0.01-0.05	0.01-0.07
L-LDL free cholesterol mmol/L	0.03-0.20	0.04-0.20	0.05-0.18	0.04-0.20	0.04-0.21
S-LDL free cholesterol mmol/L	0.04-0.48	0.05-0.45	0.06-0.45	0.05-0.43	0.05-0.53
XL-HDL free cholesterol mmol/L	0.1-0.7	0.1-0.6	0.1-0.6	0.1-0.6	0.1-0.6
L-HDL free cholesterol mmol/L	0.3-0.6	0.3-0.6	0.3-0.6	0.3-0.6	0.3-0.6
S-HDL free cholesterol mmol/L	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2
XL-VLDL triglycerides mmol/L	0-0.22	0-0.17	0-0.13	0-0.17	0-0.24
L-VLDL triglycerides mmol/L	0.01-0.41	0.01-0.42	0.00-0.27	0.01-0.39	0.01-0.49
S-VLDL triglycerides mmol/L	0.03-0.16	0.02-0.16	0.02-0.13	0.01-0.15	0.02-0.19
L-LDL triglycerides mmol/L	0.11-0.24	0.10-0.24	0.11-0.26.	0.09-0.23	0.10-0.22
S-LDL triglycerides mmol/L	0.02-0.07	0.03-0.07	0.03-0.08	0.03-0.07	0.03-0.07
XL-HDL triglycerides mmol/L	0-0.02	0-0.02	0-0.02	0-0.02	0-0.03
L-HDL triglycerides mmol/L	0.01-0.03	0-0.03	0-0.02	0-0.03	0-0.04
S-HDL triglycerides mmol/L	0-0.04	0-0.03	0-0.02	0-0.03	0-0.04
XL-VLDL phospholipids mmol/L	0-0.05	0-0.05	0-0.04	0-0.05	0-0.09
L-VLDL phospholipids mmol/L	0-0.09	0-0.11	0-0.06	0-0.10	0-0.15
S-VLDL phospholipids mmol/L	0.01-0.07	0.01-0.08	0.01-0.06	0-0.08	0.01-0.10
L-LDL phospholipids mmol/L	0.08-0.33	0.08-0.32	0.09-0.33	0.07-0.30	0.08-0.35
S-LDL phospholipids mmol/L	0.08-0.87	0.12-0.84	0.12-0.80	0.12-0.80	0.12-1.01
XL-HDL phospholipids mmol/L	0.35-2.27	0.33-2.22	0.32-1.97	0.30-2.21	0.34-2.37
L-HDL phospholipids mmol/L	2.65-3.92	2.48-3.94	2.61-3.79	2.44-3.95	2.56-4.01
S-HDL phospholipids mmol/L	0.74-1.18	0.72-1.19	0.76-1.14	0.72-1.19	0.67-1.21

Note: This table presents the reference intervals for all measurands in serum and heparinized plasma. HP all dogs n = 269, S all dogs n = 865, S puppy n = 152, S adult n = 545, S senior n = 168.

Abbreviations: BCAA, Branched-chain amino acids; HP, heparinized plasma; L-HDL, large HDL particles; L-LDL, large LDL particles; L-VLDL, large VLDL particles; S, serum; S-HDL, small HDL particles; S-LDL, small LDL particles; S-VLDL, small VLDL particles; XL-HDL, very large HDL particles; XL-VLDL, Chylomicrons and very large VLDL particles.

the same type are generally comparable. Since sample type characteristics, such as prolonged contact with RBCs in serum, caused slightly different results in serum and plasma, RIs for the sample type in question should be used. It should also be noted that glycine and pyruvate cannot be quantified in EDTA plasma, and glutamine is not quantifiable from oligofin oligomer gel tubes.

Limitations of this study are that the precision and interference studies did not include samples at clinical decision levels for all measurands. Due to the extensiveness of the NMR platform, acquiring these samples would have been extremely difficult. The sample numbers, especially in heparinized plasma, were a limitation of the storage study. The interference study for hemolysis and lipemia should be continued with a dose-response series of interferent concentrations.[15,30] Limitations of the method comparison study include the lack of a reference method, generating results that could be viewed as true measurand values.

Linearity was not assessed since NMR is considered a primary ratio method. Its linearity is considered inherently outstanding since signal intensity is directly proportional to the number of nuclei causing the specific resonance. Detection limits were not determined since low concentrations of the included measurands are not considered clinically significant.

Owing to the advantages of quantitative results, high throughput, and reproducibility, the developed NMR-based metabolomics platform validated in this study holds great potential for numerous clinical and research applications in veterinary medicine. The performance of the NMR testing platform is generally outstanding, and routine blood drawing and processing guidelines ensure the integrity of the results. The established metabolomics panel includes a wide representation of measurands from various molecular groups, including amino acids, fatty acids, glycolysis-related metabolites, and lipoproteins, enabling the comprehensive evaluation of the metabolic state of an individual.

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DISCLOSURE

CO, KV, and JP are employees, and HL, an owner and chairman of board of PetBiomics Ltd, a diagnostic laboratory company providing NMR metabolomics analyses for dogs.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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