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RESEARCH ARTICLE

Plasma metabolome reveals altered oxidative stress, inflammation, and amino acid metabolism in dogs with idiopathic epilepsy

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Abstract

Objective: Idiopathic epilepsy (IE) is the most common chronic neurological disease in dogs and an established natural animal model for human epilepsy types with genetic and unknown etiology. However, the metabolic pathways underlying IE remain largely unknown.

Methods: Plasma samples of healthy dogs ($n = 39$) and dogs with IE ($n = 49$) were metabolically profiled ($n = 121$ known target metabolites) and fingerprinted ($n = 1825$ untargeted features) using liquid chromatography coupled to mass spectrometry. Dogs with IE were classified as mild phenotype (MP; $n = 22$) or drug-resistant (DR; $n = 27$). All dogs received the same standard adult maintenance diet for a minimum of 20 days (35 ± 11 days) before sampling. Data were analyzed using a combination of univariate (one-way analysis of variance or Kruskal–Wallis rank sum test), multivariate (*limma*, orthogonal partial least squares–discriminant analysis), and pathway enrichment statistical analysis.

Results: In dogs with both DR and MP IE, a distinct plasma metabolic profile and fingerprint compared to healthy dogs was observed. Metabolic pathways involved in these alterations included oxidative stress, inflammation, and amino acid metabolism. Moreover, significantly lower plasma concentrations of vitamin B6 were found in MP ($p = .001$) and DR ($p = .005$) compared to healthy dogs.

Significance: Our data provide new insights into the metabolic pathways underlying IE in dogs, further substantiating its potential as a natural animal model for humans with epilepsy, reflected by related metabolic changes in oxidative stress metabolites and vitamin B6. Even more, several metabolites within the uncovered pathways offer promising therapeutic targets for the management of IE, primarily for dogs, and ultimately for humans.

KEYWORDS

epilepsy, metabolomics, pyridoxine, seizure

Lieselot Y. Hemeryck and Myriam Hesta contributed equally to this work.

1 | INTRODUCTION

Dogs are an established natural animal model for epilepsy types with a genetic as well as unknown etiology in humans, as they suffer from similar naturally occurring neurological diseases as humans, idiopathic epilepsy (IE) most importantly.[1] Canine and human epilepsy share clinical traits, such as the occurrence of status epilepticus and behavioral comorbidities.[2] Moreover, a similar prevalence of epilepsy is reported in dogs (i.e., .50%–.82%) in first-line practices[3] in comparison to a lifetime prevalence of .64% in humans.[4] With respect to the underlying pathophysiology, similar electrophysiological and pharmacological properties of human and canine epilepsy have been observed.[1] Lastly, environmental circumstances for pets are highly comparable to humans, because pets and their owners mostly share the same home.[5] As in humans with epilepsy, management with antiseizure medication (ASM) is inadequate in one third of dogs suffering from IE,[6] resulting in a need for supplementary therapeutic targets. New discoveries in canine epilepsy studies therefore have the potential to benefit both veterinary and human medicine.

Metabolomics is a promising research technology, with applications increasing rapidly in both human and veterinary medicine.[7] Metabolomics enables characterization of an individual's biological phenotype, thereby reflecting the integration of host-specific factors such as disease and the microbiome, and external factors including environmental influences and diet.[8] As such, the metabolome can provide insights into multiple pathophysiological processes like neuroinflammation[9] and support the elucidation of diagnostic and prognostic biomarkers or therapeutic targets[10] in epilepsy.

Canine epilepsy studies have an important advantage compared to human epilepsy trials. A standardized dietary background can be easily established by feeding all dogs identical commercial food. Thereby, nutritional inadequacies can be excluded, by providing a qualitative balanced diet fulfilling nutritional requirements for adult dogs. In metabolomics studies particularly, nutrition is one of the most important environmental influences.[11] Therefore, the standardized nutritional approach is highly beneficial for the interpretation of metabolic alterations in dogs with IE compared to healthy dogs. The combination of this standardized approach, which would be challenging in human studies, and the high translational potential of canine IE, can therefore enhance insights into epilepsy pathophysiology in humans too.

Key points

- Dogs with IE show a distinct plasma metabolome compared to healthy dogs.
- Metabolic alterations in the plasma of dogs with IE can be ascribed to oxidative stress, inflammation, and amino acid metabolism.
- Dogs with IE have significantly lower plasma vitamin B6 concentrations compared to healthy dogs.

2 | MATERIALS AND METHODS

2.1 | Study design and subjects

The study protocol (Figure 1), approved by the ethical committee of the Faculties of Veterinary Medicine and Bioscience Engineering (EC2020-091), included three groups of client-owned dogs: healthy, mild phenotype (MP), and drug-resistant (DR) IE cases. Inclusion criteria comprised unremarkable anamnesis, normal physical and neurological examinations, and normal blood and urine tests. For IE dogs, criteria included a history of IE in an otherwise healthy dog, although liver enzyme or electrolyte alterations without clinical relevance due to ASM were allowed. Body weight and body condition score (BCS) were recorded on a 9-point scale, with 4 and 5 being ideal.[12] No antibiotics were permitted for 3 months before sample collection. IE diagnosis followed the International Veterinary Epilepsy Taskforce guidelines, namely, tier I (based on signalment, medical history, video evaluation of a seizure, and routine blood examination) or tier II (tier I + bile acid, magnetic resonance imaging, and cerebrospinal fluid [CSF] evaluation).[13] An epileptic seizure diary for minimally 3 months prior to the study start was required. Dogs were then categorized as DR (failure to achieve >50% seizure reduction despite ≥2 ASMs for ≥2 months) or MP, including dogs with ≤1 seizure/3 months and no status epilepticus or cluster seizures, and dogs with a good response to ASM, that is, >50% reduction in seizure frequency post-ASM adjustment.[14]

Dogs were fed an adult maintenance diet (Purina Proplan Medium Adult with Optibalance) and treats (Purina Proplan Dental Probar) based on maintenance energy requirement (MER) per European Pet Food Industry Federation (FEDIAF) 2019 guidelines, with strict adherence to the provided diet for at least 20 days. An aliquot of this food was sent to an external laboratory (Eurofins

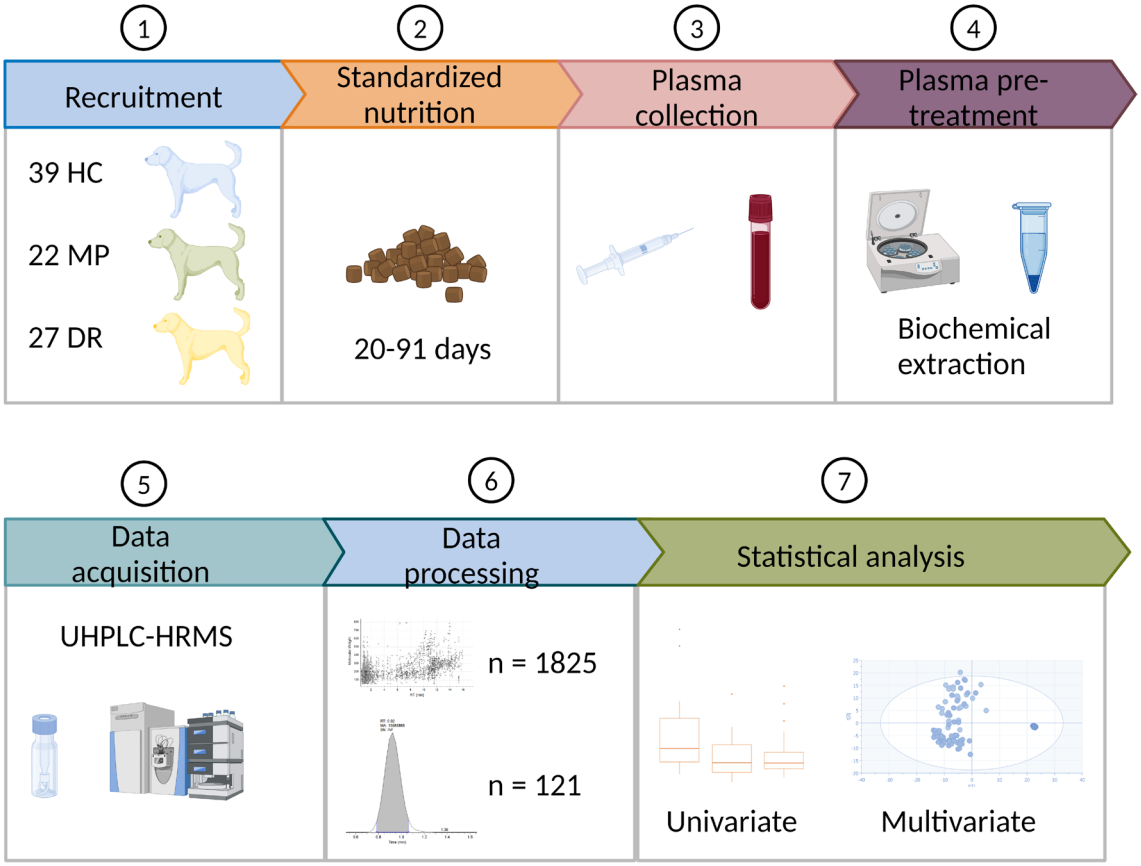

FIGURE 1 Study protocol. DR, drug-resistant; HC, healthy control; MP, mild phenotype; UHPLC-HRMS, ultra-high-performance liquid chromatography coupled to hybrid quadrupole–Orbitrap high-resolution mass spectrometry.

Testing Denmark) for vitamin B6 quantification when the trial was completed. Preprandial plasma samples were collected after an 8-h fast, centrifuged, aliquoted, flash frozen using liquid nitrogen, and stored at −80°C until ultra-high-performance liquid chromatography–high-resolution mass spectrometry analysis as per De Paepe et al.[15] Standard mixtures of 371 metabolites (Table S1) were injected before and after randomized sample analysis. Quality control samples (QCs; i.e., a pool of 70 randomly selected biological samples) and negative control (i.e., a blank sample prepared following the extraction protocol) were injected to correct instrumental drift in the beginning of the sequence, following every 10 biological samples, as well as upon finalization of the analysis. Instrumental parameters followed previously established protocols.[15]

2.2 | Data processing and statistical analysis

Clinical parameters were described as a proportion (categorical data) or mean ± SD and further analyzed using SPSS (IBM SPSS Statistics for Windows, version 29.0.2.0). Three-group comparison was performed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey test for normally distributed data or Kruskal–Wallis rank sum test for nonnormally distributed data. Categorical data were compared using a Pearson chi-squared test. IE characteristics included type of epileptic seizures (generalized tonic–clonic seizure [GTCS] or focal seizure),[16] presence of cluster seizures or status epilepticus in the complete medical history, age at seizure onset, time between sampling and last seizure, and mean seizure frequency (MSF) in the 3 months preceding sample collection. These were described as median (interquartile range), mean ± SD, or proportion. Numerical data were compared between MP and DR using an independent two-sided *t*-test or Mann–Whitney *U*-test. For all statistical tests, a *p*-value <.05 was considered significant.

Both targeted metabolites and untargeted features were normalized using internal QCs (iQCs). After iQC normalization, only metabolites/features with a coefficient of variance < 30% in the QCs were retained for further analysis.

2.2.1 | Targeted metabolomics

For the in-house available metabolite standards, peak areas were obtained by manual integration using Xcalibur 4.1 (Thermo Fisher Scientific). Only metabolites with a signal to noise ratio of >3 in 90% of the samples were considered. Identification was achieved based on accurate mass (*m/z*-value, considering both the molecular ion and C_{13}-isotope) and relative retention time (level 1 identification according to the Metabolomics Standards Initiative [MSI]).[17] Further data processing was executed using Excel (Microsoft) and R (version 4.2.3).[18]

First, plasma metabolite levels were semiquantitatively compared between groups (i.e., healthy, IE, IE MP, and IE DR). The current study aimed to identify data-driven hypotheses to guide future research for canine IE. Therefore, only metabolites significantly altered using both univariate and multivariate statistical analysis for a given pairwise comparison were retained for biological interpretation. Details regarding all significantly altered metabolites can be consulted in Table S2.

For the univariate approach, normality was evaluated with a Shapiro–Wilk test to determine which statistical test should be applied. Three-group comparison (healthy vs. MP vs. DR) was performed using a one-way ANOVA for normally distributed metabolites or a Kruskal–Wallis rank sum test for nonnormally distributed metabolites. Three-group comparison was then followed by a post hoc Tukey test following one-way ANOVA, or Dunn test following Kruskal–Wallis rank sum test to evaluate the significance of each metabolite in each pairwise comparison (i.e., healthy vs. DR, healthy vs. MP, and DR vs. MP).[19] For the multivariate approach, iQC-normalized data were log transformed and Pareto scaled using "linear models for microarray data" (*limma*).[20] Results were corrected for the confounders age, gender, and BCS. False discovery rate (FDR) correction using Benjamini–Hochberg method was applied to the obtained *p*-values, respectively indicated as P_{uni} and P_{lim}. If biologically relevant, metabolite ratios and/or Pearson correlation coefficients were calculated to assess the relationship between specific metabolites. Ratios were then compared with one-way ANOVA and post hoc Tukey test.

2.2.2 | Untargeted metabolomics

Untargeted data preprocessing was performed with Compound Discoverer 3.3 (Thermo Fisher Scientific), combining positive and negative ions. Detected features were characterized by *m/z*-value (peak intensity threshold = 500 000 arbitrary units, mass tolerance = 5 ppm), retention time (RT; maximum RT shift = .4 min), and peak intensity (minimal signal to noise ratio = 3). Data preprocessing included log transformation and Pareto scaling. Further data processing was executed in Simca 17.1 (Umetrics) and MetaboAnalyst 6.0.[21] In Simca, first, both ionization modes were combined to build an unsupervised multivariate principal component analysis (PCA-X) model including the QCs for exploration of inherent sample and QC clustering. These data were then modeled using supervised orthogonal partial least squares–discriminant analysis (OPLS-DA), whereby four pairwise comparisons were made: healthy versus IE, healthy versus DR, healthy versus MP, and DR versus MP. The model characteristics $R^2(Y)$ for fit, $Q^2(Y)$ for predictivity (both >.5), cross-validated ANOVA ($p < .05$), and permutation testing ($n = 100$) were assessed to evaluate model validity. The discriminative quality of features was investigated based on variance importance in projection scores > 2, S-plot correlations |p(corr)| > .6, and jackknifed confidence intervals not across zero. In parallel, pathway enrichment analysis using Mummichog and GSEA algorithms in MetaboAnalyst 6.0 was performed for each ionization method separately (without additional filtering steps in the MetaboAnalyst environment). Putative metabolite identification was pursued whenever possible, by matching measured *m/z*-values (<5 ppm difference) to theoretical *m/z*-values and retention time in the Chemspider or in-house database (level 2 identification according to MSI).[17]

3 | RESULTS

3.1 | Clinical characteristics of the enrolled dogs

We enrolled 39 healthy and 49 dogs diagnosed with IE, in accordance with the International Veterinary Epilepsy Taskforce guidelines[13] as tier I ($n = 36$) or tier II ($n = 13$). Of these, 22 dogs fulfilled the criteria for the MP group, and the remaining 27 dogs were categorized as DR. Within the MP dogs, 18 of 24 dogs showed a good response to ASM, three dogs required no ASM due to low MSF, and in one dog ASM was started after the first seizure, whereby the effect on MSF could not be evaluated. The total MSF for these four dogs was .08–.22 seizures/month over a follow-up period of 12–36 months. The signalment of all dogs is summarized in Table S3. The most common breeds were Border Collie ($n = 17$), crossbreed ($n = 6$), Cane Corso ($n = 5$), and Golden Retriever ($n = 5$). Of the 88 dogs, 33 were female (23 spayed) and 55 were male (25 castrated), the mean age at the start of the study was 4.70 ± 2.19 years, and the mean body weight was

26.29 ± 12.75 kg (range = 4.40–61.00 kg). No significant differences in the abovementioned clinical characteristics were observed between the three groups (Table S3). The mean BCS was 5.23 ± .99, with a significantly higher BCS found in DR IE ($p = .003$), but not MP, compared to healthy dogs.

Dogs with IE experienced a seizure frequency in the 3 months preceding sample collection between 0 and 10 seizures per month, whereby the MSF for DR dogs was 2.9 ± 2.2 seizures/month and for MP dogs .2 ± .3 seizures/month. The median time between the last epileptic seizure and plasma sampling was significantly longer ($p < .001$) for MP (i.e., 117.5 days, interquartile range = 82.8) compared to DR (i.e., 7 days).[16] The mean age at epileptic seizure onset for dogs with IE was 2.46 ± 1.46 years, whereby no significant difference was seen between MP and DR dogs (Table S3). Complete clinical history revealed the occurrence of GTCSs for all dogs, and in two dogs additional focal seizures were seen. Cluster seizures and status epilepticus were present in the history of 30 (i.e., 61.2% [19 DR and 11 MP]) and 13 dogs (i.e., 26.5% [8 DR and 5 MP]), respectively. Furthermore, 37 (23 DR and 14 MP), 22 (15 DR and 7 MP), and 11 (7 DR and 4 MP) dogs received phenobarbital, potassium bromide, or levetiracetam, respectively. Seizure characteristics and type of ASM were not significantly different between MP and DR dogs (Table S3).

3.2 | Selected metabolites differ between dogs with IE and healthy dogs

Of the 121 plasma metabolites that met our inclusion criteria, 24 and 13 metabolites, respectively, were significantly altered in the plasma of dogs with DR IE and MP IE compared to healthy dogs (Table 1). All 13 metabolites altered in the plasma of MP dogs were also altered in the plasma of DR dogs. Mostly, α-amino acids (AAs) or derivatives were significantly increased in the plasma of IE dogs compared to healthy dogs. Seven of these were only increased in DR dogs, and seven were significantly increased in the plasma of both DR and MP dogs compared to healthy dogs. Only carnosine and N,N-dimethylarginine were decreased in DR dogs compared to healthy dogs. The ratio between branched-chain AAs (BCAA) and aromatic AAs was not significantly different between healthy (.68 ± .15) and MP (.72 ± .20, $p = .62$) or DR dogs (.76 ± .19, $p = .12$). Although tryptophan as such was not significantly different between groups, N-acetyl-tryptophan was increased in both IE groups compared to healthy dogs. Moreover, two ratios with tryptophan were calculated additionally (i.e., tryptophan [Trp]/large neutral AA [LNAA] and kynurenine [Kyn]/Trp ratio). These were not significantly different between healthy (Trp/LNAA = .19 ± .06 and Kyn/Trp = 1.033 ± .28) and MP (Trp/LNAA = .18 ± .08, $p = .95$; Kyn/Trp = 1.0 ± .37, $p = .94$) or DR (Trp/LNAA = .15 ± .04, $p = .12$) dogs. However, the Kyn/Trp ratio was significantly decreased in DR (.86 ± .21, $p = .05$) compared to healthy dogs. Besides AA alterations, two glucose-derived acids were increased in the plasma of IE dogs, in both MP and DR compared to healthy dogs (i.e., gluconic acid and glucuronic acid; Table 1). Two additional metabolites were increased in the plasma of DR compared to healthy dogs, but not in MP dogs (i.e., 4-guanidinobutanoic acid [4GBA] and xanthurenic acid). Conversely, ethyl-3-indole acetic acid, a tryptophan-derived metabolite, was decreased in the plasma of IE dogs, in both MP and DR compared to healthy dogs, and 2,6-dihydroxybenzoic acid was significantly decreased in the plasma of DR compared to healthy dogs, but not in MP dogs.

In the comparison between DR and MP, no metabolites were found significantly altered in both univariate and multivariate analysis, after FDR correction. Therefore, multivariate analysis before FDR correction combined with univariate analysis (with FDR correction) was evaluated additionally. Hereby, only 4GBA was significantly increased in the plasma of DR dogs compared to MP dogs ($P_{uni} = .014$, nonadjusted $P_{lim} = .004$). Moreover, it was also significantly increased in DR but not in MP dogs compared to healthy dogs (Table 1).

3.3 | Metabolic fingerprints discriminate plasma of dogs with IE from healthy dogs

Untargeted data processing resulted in 920 positive features and 905 negative features. First, an unsupervised PCA-X model was built, whereby clustering of the healthy versus IE plasma samples visibly indicated a distinct plasma metabolome in IE dogs compared to healthy dogs, and the distinction from DR dogs was even more pronounced (Figure 2A).

Three OPLS-DA models were compliant with the set validation criteria (Figure 2B–D); however, no valid model could be built to discriminate the plasma metabolome of MP and DR dogs. From the validated OPLS-DA models, 21 features discriminated both DR and MP, and an additional 16 only discriminated plasma of DR dogs from healthy dogs. Three of the discriminative features were putatively identified (level 2 identification based on MSI[17]). These comprised hydroxyphenobarbital, for which a peak was only present in samples from dogs receiving phenobarbital. Also a phenylpropane (i.e. 2,6-di-tert-butyl-benzenediol) and a fatty acyl glycoside (i.e., ethyl-7-epi-12-hydroxyjasmonate glucoside) were higher in plasma from both MP and DR versus healthy dogs, which was more pronounced in DR dogs.

TABLE 1 Significantly altered metabolites with their iQC normalized peak areas and corresponding *p*-values.

Metabolite	*m/z*	RT, min	Ion	iQC normalized peak areas			Adjusted *p*, DR vs. HC		Adjusted *p*, MP vs. HC	
				Healthy, mean ± SD	IE MP, mean ± SD	IE DR, mean ± SD	P_{uni}	P_{lim}	P_{uni}	P_{lim}
2.6-Dihydroxybenzoic acid	153.01933	8.8	[M−H]−	1.199 ± .605	.993 ± .725	.683 ± .556	**.003**	**.037**	.165	**.000**
4-Guanidinobutanoic acid	146.0924	1.33	[M+H]+	.793 ± .545	.841 ± .617	1.425 ± 1.028	**.009**	**.009**	.991	**.000**
Carnosine	227.114	.92	[M+H]+	1.145 ± .372	.943 ± .397	.793 ± .204	**.000**	**.004**	.069	.120
D-α/L2-Aminobutyric acid	104.07061	1	[M+H]+	.908 ± .321	1.222 ± .741	1.264 ± .524	**.004**	**.031**	**.048**	.110
Gluconic acid	195.05103	.96	[M−H]−	.151 ± .057	1.536 ± 1.706	2.215 ± 2.481	**.000**	**.000**	**.000**	**.000**
Glucuronic acid	193.03547	1	[M−H]−	.405 ± .273	1.439 ± 1.188	1.786 ± 1.428	**.000**	**.000**	**.000**	**.000**
Ethyl-3-indole acetic acid	204.10191	11.71	[M+H]+	1.865 ± 1.515	.932 ± .726	.845 ± .675	**.007**	**.022**	**.021**	**.049**
γ-Glutamyl-phenylalanine	295.12884	7.93	[M+H]+	.803 ± .233	1.390 ± .679	1.268 ± .49	**.000**	**.004**	**.000**	**.000**
Glutamic acid	148.06043	.94	[M+H]+	.925 ± .329	1.096 ± .397	1.307 ± .447	**.001**	**.004**	.168	.277
Leucine	132.10191	2.28	[M+H]+	.974 ± .166	1.192 ± .344	1.137 ± .205	**.007**	**.047**	**.008**	**.020**
Phenylalanine	166.08626	4.8	[M+H]+	.881 ± .150	1.234 ± .426	1.191 ± .318	**.000**	**.003**	**.000**	**.001**
Lysine	147.1128	.75	[M+H]+	.854 ± .280	1.052 ± .350	1.247 ± .444	**.000**	**.002**	.056	.110
N-Acetyl-asparagine	175.07133	1.14	[M+H]+	.771 ± .377	1.120 ± .500	1.375 ± .543	**.000**	**.000**	**.016**	**.030**
N-Acetyl-DL-tryptophan	247.1077	10.37	[M+H]+	.944 ± .496	1.448 ± .707	1.388 ± .596	**.009**	**.042**	**.005**	**.020**
N-Acetyl-L-methionine	192.06889	7.55	[M+H]+	.838 ± .221	1.396 ± .668	1.299 ± .487	**.000**	**.002**	**.000**	**.000**
N-Acetyl-L-tyrosine	224.09173	7.6	[M+H]+	.863 ± .428	1.378 ± .719	1.488 ± .619	**.000**	**.008**	**.003**	**.027**
N-Acetyl-leucine	174.11247	9.59	[M+H]+	.859 ± .365	1.410 ± .788	1.350 ± .423	**.000**	**.011**	**.002**	**.007**
N-Acetyl-glutamic acid	188.05537	1.75	[M−H]−	.861 ± .286	1.374 ± .715	1.332 ± .591	**.002**	**.007**	**.004**	**.007**
N-Methyl-aspartic acid	148.06043	.89	[M+H]+	.877 ± .315	1.034 ± .325	1.301 ± .519	**.001**	**.003**	.138	.276
N,N-Dimethylglycine	104.07082	1	[M+H]+	.907 ± .317	1.276 ± .838	1.265 ± .497	**.001**	**.031**	**.028**	.078
N,N-Dimethylarginine	203.15025	1.02	[M+H]+	1.490 ± .437	1.135 ± .503	1.046 ± .501	**.002**	**.037**	**.012**	.120
N6-Acetyl-L-lysine	189.12337	1.39	[M+H]+	.898 ± .285	1.137 ± .353	1.172 ± .487	**.026**	**.028**	**.035**	**.049**
Threonine	120.06574	.94	[M+H]+	.766 ± .273	1.102 ± .467	1.287 ± .571	**.000**	**.001**	**.005**	.059
Xanthurenic acid	206.04478	7.25	[M+H]+	.935 ± .835	1.480 ± 1.367	1.713 ± 1.100	**.015**	**.042**	.217	.199

Note: Significant *p*-values are in bold, and metabolites significantly altered in both MP and DR IE are underlined. Univariate three-group comparison (healthy vs. MP vs. DR) was performed using a one-way analysis of variance for normally distributed metabolites or a Kruskal–Wallis rank sum test for nonnormally distributed metabolites, followed by a post hoc Tukey or Dunn test, respectively, and FDR correction using BH. Multivariate three-group comparison was performed using linear models for microarray data (*limma*) on log transformed and Pareto scaled data. The multivariate model included confounders age, gender, and body condition score and was followed by FDR correction using BH. Normalized peak areas were calculated as the ratio between peak area of the metabolite in the biological sample and mean peak area of the metabolite in the proceeding quality control samples, and therefore they do not have a unit.

Abbreviations: BH, Benjamini–Hochberg method; DR, drug-resistant; FDR, false discovery rate; IE, idiopathic epilepsy; iQC, internal quality control sample; *m/z*, mass to charge ratio; MP, mild phenotype; P_{lim}, *p*-value from multivariate statistical approach (i.e., *limma*); P_{uni}, *p*-value from univariate statistical approach; RT, retention time.

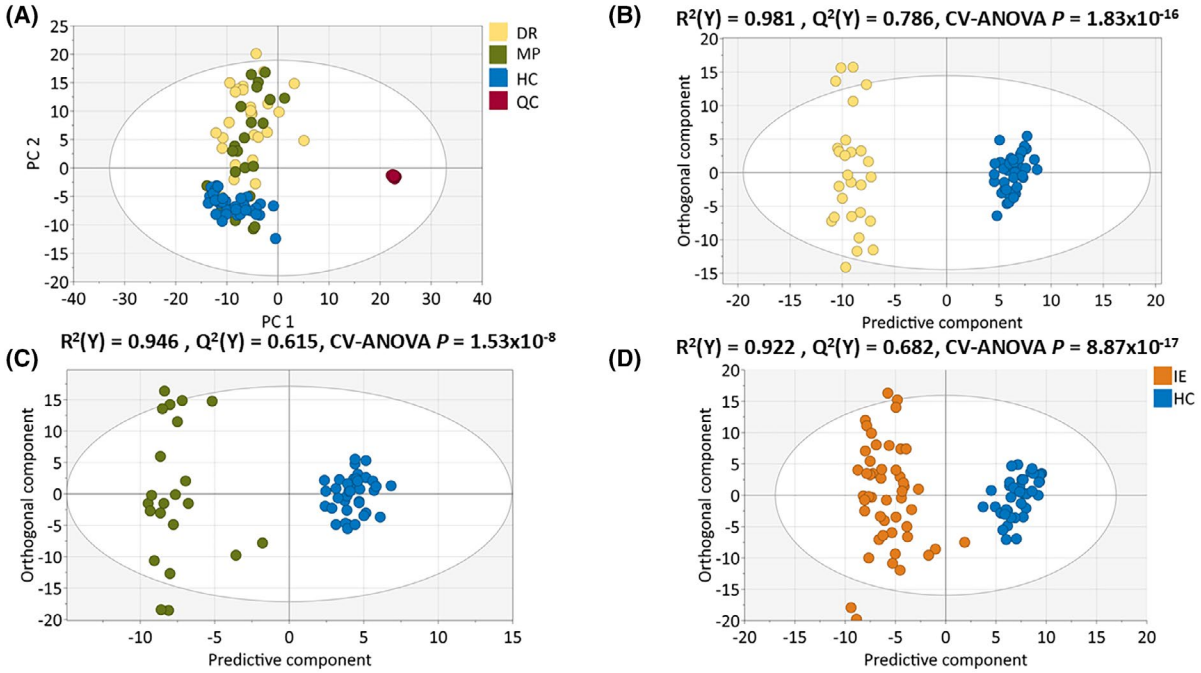

FIGURE 2 Score plots of the plasma metabolic fingerprints, with each dot representing the fingerprint of an individual dog. Untargeted data were internal quality control sample normalized, log transformed, and Pareto scaled prior to plotting. (A) PCA-X, (B) orthogonal partial least squares–discriminant analysis OPLS-DA of healthy (HC) versus DR, (C) OPLS-DA of HC versus MP, (D) OPLS-DA of HC versus IE. CV-ANOVA, cross-validated analysis of variance; DR, drug-resistant IE; HC, healthy control dog; IE, idiopathic epilepsy (DR and MP); MP, mild phenotype IE; PC, principle component; QC, quality control.

Pathway enrichment analysis of the positive ionized features revealed a trend toward perturbation of the cysteine and methionine ($p = .060$) pathways, D-AA ($p = .066$) and valine, leucine and isoleucine degradation ($p = .066$), and vitamin B6 ($p = .060$) metabolism in dogs with IE compared to healthy dogs (Figure 3A). For the latter, a confirmatory quantitative analysis was performed to substantiate our findings. Vitamin B6 concentrations (nmol·L^{-1}) were determined in their active form (i.e., pyridoxal-5-phosphate [PLP]) in available sample leftovers ($n = 88$) via tandem mass spectrometry by an external accredited laboratory (i.e., Algemeen Medisch Laboratorium). Hereby, a significant decrease was seen in both MP (280.32 ± 59.88 nmol·L^{-1}, $p = .001$) and DR (275.93 ± 48.74 nmol·L^{-1}, $p = .005$) compared to healthy dogs (623.05 ± 67.19 nmol·L^{-1}; Figure 3B).

4 | DISCUSSION

Our study revealed a distinct plasma metabolic profile and fingerprint for DR and MP IE compared to healthy dogs. It is noteworthy that the plasma of IE dogs revealed a distinct AA metabolism, together with alterations in metabolites related to oxidative stress, including gluconic and xanthurenic acid and 4GBA. Interestingly, both altered pathways could be attributed to a decreased vitamin B6 plasma level in dogs with IE. The study aimed to deepen understanding of IE pathophysiology, with detailed biological interpretation of each metabolite provided in the following discussion, including a schematic overview of the plasma AA metabolism alterations in dogs with IE (Figure 4). However, it should be noted that the current study design cannot distinguish between potential causes or consequences of epileptic seizures.

4.1 | Markers of oxidative stress and inflammation are increased in the plasma of dogs with IE

Our study demonstrated increased gluconic (DR and MP) and xanthurenic acid (only DR) plasma levels in dogs with IE, indicating similar metabolic alterations as described in humans. In the formation process of gluconic acid, hydrogen peroxide is released,[22] and as such it is considered a marker for oxidative stress. Recently, increased gluconic acid levels were associated with hyperglycemia and cytotoxic brain injury in 381 human patients with acute stroke, highlighting its importance in the brain's oxidative stress metabolism.[23] In the same study, higher levels of xanthurenic acid could also be observed alongside

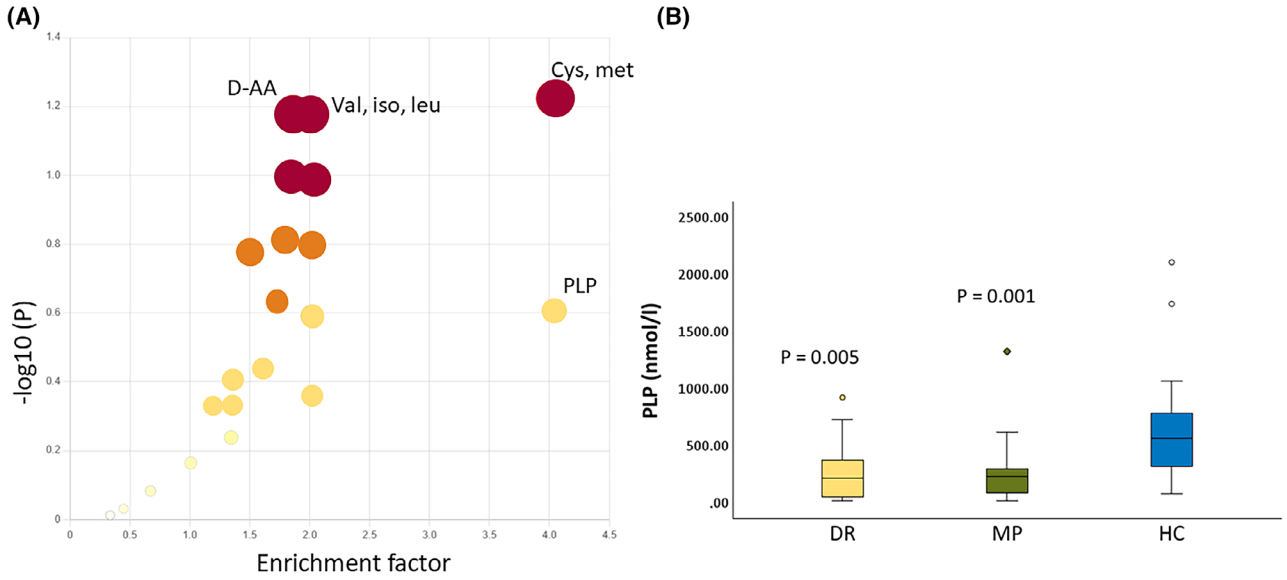

FIGURE 3 (A) Pathway enrichment summary plot whereby all matched pathways are presented as circles. The color and size of each circle corresponds to its *p*-value and enrichment factor, respectively. The enrichment factor of a pathway is calculated as the ratio between the number of significant pathway hits and the expected number of compound hits within a pathway. Cys, met, cysteine and methionine metabolism; D-AA, D-amino acid metabolism; PLP, pyridoxal-5-phosphate or vitamin B6 metabolism; Val, iso, leu, valine, isoleucine, and leucine degradation. (B) Boxplots representing the measured concentration of PLP in each group. Bonferroni corrected *p*-values (Kruskal–Wallis rank sum test) for the pairwise comparisons with HC plasma are provided. A decreased PLP concentration in DR ($n = 27$) and MP ($n = 22$) compared to healthy dogs ($n = 38$) is portrayed. DR, drug-resistant idiopathic epilepsy; HC, healthy control; MP, mild phenotype idiopathic epilepsy.

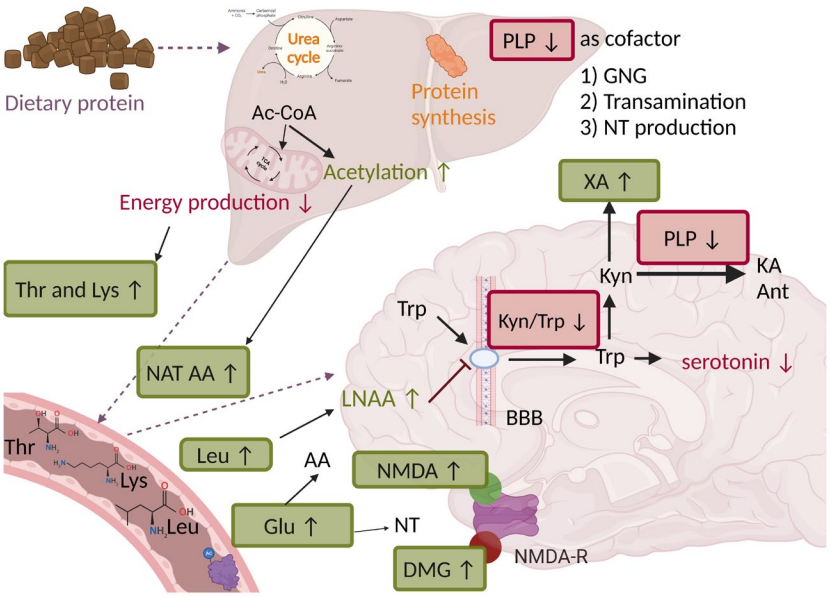

FIGURE 4 AA metabolism in dogs with idiopathic epilepsy. Only 23% of the AA obtained from dietary protein is ultimately transferred to the systemic circulation. In the liver, 57% is typically used for the urea cycle and 20% for protein synthesis. In the plasma of dogs with idiopathic epilepsy, alterations in multiple AAs were seen in this study, implicating potential alterations in the AA hepatic and brain metabolism. The alterations found in plasma and their hypothesized effect on the hepatic and brain metabolism are displayed as framed text in the picture. AA, amino acid; Ac-CoA, acetyl-coenzyme A; Ant, anthranilic acid; BBB, blood–brain barrier; DMG, N,N-dimethyl-glycine; Glu, glutamate; GNG, gluconeogenesis; KA, kynurenic acid; Kyn, kynurenine; Leu, leucine; LNAA, large neutral amino acids; Lys, lysine; NAT AA, N-acetylated amino acids; NMDA, N-methyl-D-aspartate; NMDA-R, NMDA receptor; NT, neurotransmitter; PLP, pyridoxal-5-phosphate (i.e., vitamin B6); Thr, threonine; Trp, tryptophan; XA, xanthurenic acid.

hyperglycemia in stroke patients.[23] Inflammatory upregulation of the kynurenine pathway can lead to functional vitamin B6 deficiencies, despite adequate nutritional intake. Vitamin B6 deficiency, in turn, reduces the conversion of kynurenine to other downstream metabolites,[24] as such causing an increase in xanthurenic acid.[25]

Our study revealed as a first of its kind significantly lower vitamin B6 concentrations in both MP and DR IE compared to healthy dogs. In young children, however, multiple vitamin B6-dependent epilepsy types are well known.[26] The gold standard in these epilepsy types comprises oral pyridoxine supplementation,[27] with dosages 170–1000-fold the recommended dietary allowances for healthy people.[28] More recently, the role of vitamin B6 in adults experiencing seizures[29] and a possible role for oxidative stress[30] were highlighted. Therefore, pyridoxine supplementation might aid in improving seizure control for dogs with IE too. However, high vitamin B6 intake could induce toxicity (i.e., peripheral neuropathy) in both dogs[31] and humans.[32] Therefore, the mechanism of action and optimal dosages should be further established for canine IE specifically. The food in the current study contained .40 ± .06 mg/100 kcal vitamin B6, exceeding the recommended vitamin B6 levels for complete dog food established by the FEDIAF 2024 guidelines (i.e., .036 mg/100 kcal). The increased plasma xanthurenic acid and decreased vitamin B6 detected in our study are therefore suspected to be a consequence of increased utilization rather than nutritional deficiency, given the standardized balanced nutritional background.

We also observed a significant decrease in carnosine and 2,6-dihydroxybenzoic acid in the plasma of DR compared to healthy dogs. Carnosine is a multipotent protector against oxidative damage and is moreover attributed neuroprotective effects when orally supplemented.[33] The decrease in 2,6-dihydroxybenzoic acid is likely related to alterations in intestinal microbiota, as it is a diet-associated metabolite, resulting from intestinal microbial metabolization of dietary phenols.[34] Hydroxybenzoic acids can have multiple functional properties, whereby 2,6-dihydroxybenzoic acid specifically shows little antioxidative, but also prooxidative, activity together with antimicrobial activity in an in vitro cell line.[35]

Considering the increases observed in markers for inflammation and oxidative stress (i.e., gluconic acid and xanthurenic acid), together with the decrease in carnosine and 2,6-dihydroxybenzoic acid, an altered oxidative stress metabolism is hypothesized in dogs with IE compared to healthy dogs, similar to what is described in epilepsy research in humans[36] and in other animal models.[37]

Finally, 4GBA showed significantly higher plasma levels in DR IE dogs compared to healthy dogs and MP IE dogs, indicating a potential as future biomarker for the response to ASM in dogs with IE. The presence of 4GBA in the mammalian brain has been reported,[38] whereby accumulation induced epileptic discharges in rats, rabbits, and cats and is therefore considered convulsive.[39] 4GBA is a guanidino compound and substrate for the blood–brain barrier (BBB) creatine transporter. Increased plasma levels of 4GBA could interfere with creatine uptake in the brain and therefore alter the brains' energy homeostasis, leading to a reduced seizure threshold.[40] Furthermore, in vitro data showed a proinflammatory effect of 4GBA on monocytes and granulocytes,[41] with possible mirror effect in the brain, whereby inflammatory mediators and cytokines contribute to epileptogenesis.[42] Our data therefore aid to bridge the gap between experimental animal, in vitro studies, and clinical research.

4.2 | Plasma of dogs with IE showed a distinct AA metabolism

An alteration in multiple AAs was observed in the fasted plasma of dogs with IE, together with trends toward disruption of cysteine and methionine metabolism, branched chain AA degradation, and D-AA metabolism in the pathway enrichment analysis. D-AAs comprise only a small proportion of the total pool in living organisms, because the L-form is more dominant.[43] Our analytical methodology does not reliably discriminate between D- and L-AAs as evaluated, using both enantiomers of the leucine, glutamic acid, and alanine analytical standards. Consequently, we hypothesize that the alterations as uncovered through pathway analysis are more likely to be related to the general AA metabolism, rather than the specific D-enantiomers.

An increase in four essential AAs (i.e. lysine [DR IE], phenylalanine, threonine, and leucine [MP and DR IE]) was noted in the plasma of dogs with IE compared to healthy dogs. Lysine and threonine are typically used in the liver for energy production via ketogenic pathways or oxidative phosphorylation, respectively.[44] An accumulation of threonine in the plasma of IE dogs could therefore be related to decreased oxidative glucose metabolism, which has been shown previously in chronic epilepsy rodent models.[45] Interestingly, oxidative stress, as we observed to be increased in dogs with IE, may impair oxidative glucose metabolism by inactivating key enzymes in oxidative phosphorylation.[45]

The observed trends in AA ratios suggest potential alterations in the Trp uptake via the BBB and metabolism.[46] Therefore, the Kyn/Trp ratio was evaluated, whereby a significant decrease in DR compared to healthy dogs was observed. Inflammatory upregulation

of the Kyn to xanthurenic acid conversion[25] could lie at the origin of this decreased ratio. Increased BCAAs like leucine are shown to lower the brain uptake of tryptophan and tyrosine, which could lead to reduced serotonin and catecholamine synthesis.[47] Serotonin is a neurotransmitter modulating a wide range of neuropsychological processes.[48] Moreover, it plays a critical role in the pathophysiology of epilepsy and sudden unexpected death in people with epilepsy.[49] In the conversion of tryptophan to serotonin, vitamin B6 acts as a coenzyme.[50] However, vitamin B6 as such was decreased in the plasma of IE compared to healthy dogs. As a result, we may conclude that an indirect decrease of serotonin, related to increased plasma leucine, a tendency for reduced uptake of Trp via the BBB, and decreased vitamin B6 may contribute to the pathophysiology of IE.

Ethyl-3-indole acetic acid, a member of the indole-3 acetic acids (IAA), and a typically intestinal bacteria-derived tryptophan metabolite, was also decreased in the plasma of dogs with IE, even more pronounced in DR dogs. Supplementation of IAA in a mouse model of depression and anxiety suggested a positive effect on brain function by increasing brain-derived neurotrophic factor expression in the hippocampus and decreasing hypothalamus–pituitary–adrenal axis overactivation. As such, IAA prevented the decline of hippocampal serotonin and dopamine.[51] In contrast, a study in humans with chronic kidney disease receiving hemodialysis showed a correlation between IAA levels and poor outcome in cognitive tests, suggesting a negative effect on brain function.[52] It may be hypothesized that IAA levels need to be within specific margins for optimal brain functionality, whereby IAA levels that are either too low or too high could have a negative impact on brain function in animals and humans.

An alteration in three methylated AAs was also observed in the plasma of DR IE compared to healthy dogs. Both N-methyl-aspartic acid and N,N-dimethyl-glycine, increased in the plasma of DR IE dogs, can act as agonists of the N-methyl-D-aspartate receptor (NMDA-R), on the glutamate and glycine binding sites, respectively. Together, these could induce full activation of NMDA-R, as this requires both glycine and glutamate agonists.[53] The NMDA-R is crucial for neuronal viability and synaptic plasticity of neurons,[54] with a pathogenic role in brain damage induced by status epilepticus.[55] N,N-Dimethylarginine, decreased in the plasma of DR IE, is a byproduct of protein modification related to arginine.

Phenylalanine, γ-glutamyl-phenylalanine, and glutamate (detected in its ionized form, i.e., glutamic acid) were increased in the plasma of MP and DR or only DR dogs (for the latter) as compared to healthy dogs. Glutamate is one of the most abundant AAs, with a critical role in metabolism and signaling.[56] A linear correlation between CSF and blood was previously established during cerebral ischemia, in contrast to normal circumstances, whereby glutamate does not easily cross the BBB.[57] However, most of the dietary glutamate is metabolized by the intestines and does not reach peripheral circulation.[56] Moreover, CSF glutamate is known to increase in the acute phase (<48 h) after seizures in a canine model of complex partial seizures.[58] The increase in glutamate might therefore be related to the significantly shorter time between last seizure and sampling in DR compared to MP dogs. γ-Glutamyl-phenylalanine is a dipeptide of glutamic acid and phenylalanine, formed by transpeptidation between glutathione and phenylalanine, as catalyzed by γ-glutamyl-transpeptidase (GGT). Phenylalanine as such was also increased in IE compared to healthy dogs. In contrast, routine biochemistry analyses at the start showed that GGT was within the reference range for all dogs included in the study (0–7 U/L, reference range = 0–11 U/L), with no significant differences between groups ($p = .16$).

Lastly, N-acetyl-methionine, N-acetyl-leucine, N-acetyl-tyrosine, N-acetyl-asparagine, N-acetyl-tryptophan, N6-acetyl-lysine, and N-acetyl-glutamic acid were increased in the plasma of DR and MP dogs compared to healthy dogs. These acetylated AAs are formed by an acetylation process catalyzed by N-terminal acetyl transferases (NATs). NATs transfer an acetyl group derived from acetyl coenzyme A to the N-terminus of the AA, altering the biological properties of the molecule.[59] Acetylation is an important form of phase II elimination, as seen in the brain.[60] In humans, two isotypes exist, namely, NAT1 and NAT2, whereby NAT1 is mainly involved in the metabolism of endogenous metabolites and NAT2 metabolizes xenobiotics.[60] The observed increase in N-acetylated AAs in the plasma of DR dogs might be related to an alteration in NAT2 expression, possibly triggered by higher exposure to xenobiotic compounds (i.e., ASM).[38] Moreover, specific phenotypes of NAT2 have been associated with late onset Alzheimer disease[61] and familiar Parkinson disease,[62] suggesting their importance in BBB transport and brain function in humans.

4.3 | Study strengths and limitations

A standardized nutritional background was established by providing an identical diet for all subjects, fulfilling dietary requirements for both healthy controls and dogs with IE. Owners received nonbinding advice on energy intake based on MER, but actual intake was not monitored, potentially affecting nutrient intake. DR dogs exhibited a significantly higher BCS compared to healthy dogs, likely due to polyphagia, a known side effect of ASM.[63] Despite this,

BCS remained stable from start to sample collection, suggesting actual energy intake approximated targeted MER. The lack of precise monitoring of food intake is a first study limitation and should be addressed in future research.

A second limitation of our study design is the potential confounding effect of seizure characteristics, such as MSF and time between the last epileptic seizure and sampling. Metabolites altered only in the DR group, but not in MP, compared to healthy controls, may be seizure-related. Conversely, metabolites altered in both DR and MP are unlikely to be seizure-dependent. The time between the last seizure and sampling varied widely (1–1337 days), with a median of 117 days in MP dogs, excluding an acute seizure impact on their plasma metabolome. Future studies should include samples from both acute and chronic phases of seizures to clarify their influence on the plasma metabolome. Another limitation related to seizures is the use of owner diaries to monitor seizure frequency. Although this is the most commonly used method for dogs, it inherently implies that seizures might be missed, especially focal or nonmotor seizures. However, the most common seizure types in IE dogs are GTCSs or focal seizures evolving into GTCSs,[64] which is reflected in our population, where all dogs experienced GTCSs. These seizures are less likely to be missed, as owners report traces like urine, saliva, moved furniture, and/or postictal behavior when missing the GTCS itself.

Interestingly, our metabolome data led to the hypothesis that vitamin B6 could play a part in the pathophysiology. To confirm this hypothesis, a quantitative PLP analysis was performed, demonstrating the discovery potential of our metabolomics approach. Using the untargeted approach, however, no discriminative features could be matched to our in-house database. This likely indicates that there are other potentially important discriminative metabolic features that are not yet included in our targeted approach, highlighting future research opportunities to identify and clarify the role of these features.

5 | CONCLUSIONS

A distinct metabolic plasma profile and fingerprint in dogs with IE compared to healthy dogs on a standardized nutritional background were demonstrated. Metabolic differences between IE and healthy dogs were driven by alterations in pathways that could be related to vitamin B6 metabolism, oxidative stress, inflammation, and AA metabolism. Significantly lower vitamin B6 concentrations in the plasma of dogs with DR and MP IE were confirmed. To the best of our knowledge, this is the first study that shows alterations in vitamin B6 metabolism in dogs with IE, whereas in humans a genetic epilepsy type related to vitamin B6 deficiencies is well described. The acquired insights can therefore further be exploited to (1) increase our understanding of the dog as a natural animal model for different types of epilepsy in humans; (2) delve deeper into the potential causal role of vitamin B6 deficiency in epileptogenesis, primarily in dogs, and ultimately in humans; and (3) investigate oxidative stress, AAs, and vitamin B6 pathways for future therapeutic targets in the management of IE.

AUTHOR CONTRIBUTIONS

Fien Verdoodt contributed to the conception and design of the study; acquisition, analysis, and interpretation of data; and drafting of the manuscript. Lieselot Y. Hemeryck and Myriam Hesta contributed to the conception and design of the study; acquisition, analysis, and interpretation of data; and critical revision of the manuscript. Lynn Vanhaecke and Greet Junius contributed to the analysis and interpretation of the data and critical revision of the manuscript. Lieselot Y. Hemeryck, Luc Van Ham and Sofie F. M. Bhatti contributed to the interpretation of the data and critical revision of the manuscript. Jenifer Molina contributed to the critical revision of the manuscript. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

F.V. is currently working on a doctoral research project, including the current study, regarding the role of the gastrointestinal microbiome and nutrition in canine IE, which is financially supported by Nestlé Purina Petcare Europe. M.H. is a member of the advisory board of Nestlé Purina Petcare Europe. M.H. has been paid for several consulting services by a variety of pet food companies. The authors have no other financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in MetaboLights at https://www.ebi.ac.uk/metabolights/MTBLS10915, reference number MTBLS10915.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. This research was in compliance with European legislation on animal experimentation (EU directive 2010/63/EU) and the ARRIVE guidelines. The study protocol was approved by the ethical committee of the faculties of Veterinary Medicine and Bioscience Engineering, Ghent University (EC2020-091).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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