Untargeted global metabolomic profiling of healthy dogs grouped on the basis of grain inclusivity of their diet and of dogs with subclinical cardiac abnormalities that underwent a diet change

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OBJECTIVE
To compare metabolomic profiles of dogs eating grain-free (GF) versus grain-inclusive (GI) diets (1) for healthy dogs at baseline and (2) for dogs with subclinical cardiac abnormalities at 12 months after a diet change.

SAMPLE
Serum samples from 23 dogs eating GF diets and 79 dogs eating GI diets, of which 17 (8 eating a GF diet and 9 eating a GI diet) were reevaluated 12 months after a diet change.

PROCEDURES
Metabolomic profiles were developed by means of ultrahigh-performance liquid chromatography–tandem mass spectroscopy of serum samples. Baseline results for the GF group were compared with those for the GI group. Dogs from both groups with subclinical cardiac abnormalities were transitioned to a GI, pulse-free, intervention diet, and samples collected 12 months later were compared between diet groups. Statistical significance for biochemical group differences was defined as $P < .05$ with a false discovery rate ($q$) $< .10$.

RESULTS
Baseline differences in lipid metabolism and amino acid metabolism were found between the GF and GI diet groups. There were 46 metabolites that were higher and 82 metabolites that were lower in the GF group ($n = 23$), compared with the GI group ($79$). Comparison of the GF ($n = 8$) and GI ($9$) groups 12 months after the diet change showed only 6 metabolites that were higher and 11 metabolites that were lower in the GF group, compared with the GI group.

CLINICAL RELEVANCE
Metabolomic pathway differences between dogs eating GF versus GI diets highlight the important effect of diet in metabolomics analyses. The clinical importance of these differences and how they might relate to cardiac disease in dogs remains undetermined.
and pulse inclusivity have not yet been reported. A recent study applied a metabolomics approach to dog food and showed that diets seemingly associated with DCM differed biochemically from diets seemingly not associated with DCM, but similar studies of blood samples from dogs are lacking. Additionally, metabolomic profiling of dogs before and after a diet change could improve our understanding of how disease improvement or reversal occurs after a diet change, which is a unique feature of diet-associated DCM.

The objectives of the study reported were to investigate whether the biochemical footprint of apparently healthy dogs differed on the basis of diet type being consumed and to determine whether a diet change would alter the metabolomic profile in dogs with subclinical cardiac abnormalities. We hypothesized that dogs eating GF diets would differ biochemically from dogs eating GI diets and that fewer differences would be found after a diet change.

Materials and Methods

The study involved untargeted global metabolomic profiling of serum samples that had been frozen at –80 °C as part of a larger study. Sample collection had been approved by the University of Florida College of Veterinary Medicine’s Institutional Care and Use Committee (No. 201810504 and No. 202110504), and client consent had been obtained.

Briefly, healthy dogs of 3 breeds (Doberman Pinscher, Golden Retriever, and Miniature Schnauzer) that had undergone echocardiography and measurement of serum cardiac biomarker (N-terminal pro-B-type natriuretic peptide and high-sensitivity cardiac troponin I) concentrations were assigned to 2 groups on the basis of grain inclusivity of the diet (GF or GI), the dog had been eating for a minimum of 6 months before study enrollment. Serum samples were obtained at baseline from all dogs and stored at –80 °C until untargeted global metabolomic analysis was performed. Owners of dogs found to have echocardiographic abnormalities (normalized left ventricular diameter in diastole > 1.8, normalized left ventricular diameter in systole > 1.2, or fractional shortening < 25%), an N-terminal pro-B-type natriuretic peptide concentration higher than the laboratory-provided reference range (> 735 pmol/L for Doberman Pinschers and > 900 pmol/L for Golden Retrievers and Miniature Schnauzers), or a high-sensitivity cardiac troponin I concentration higher than the laboratory-provided reference range (> 0.06 ng/mL) were offered participation in the longitudinal arm of the study if the owner was willing to change the diet to 1 of 6 extruded, GI, pulse-free, intervention diets. Serum was collected from these dogs 12 months after changing to a GI, pulse-free, intervention diet were available for 17 dogs (8 that were originally eating a GI diet). Clinical details of these dogs included echocardiographic abnormalities (normalized left ventricular diameter in systole > 1.2, or fractional shortening < 25%), an N-terminal pro-B-type natriuretic peptide and high-sensitivity cardiac troponin I) concentrations were assigned to 2 groups on the basis of grain inclusivity of the diet (GF or GI), the dog had been eating for a minimum of 6 months before study enrollment. Serum samples were obtained at baseline from all dogs and stored at –80 °C until untargeted global metabolomic analysis was performed. Owners of dogs found to have echocardiographic abnormalities (normalized left ventricular diameter in diastole > 1.8, normalized left ventricular diameter in systole > 1.2, or fractional shortening < 25%), an N-terminal pro-B-type natriuretic peptide concentration higher than the laboratory-provided reference range (> 735 pmol/L for Doberman Pinschers and > 900 pmol/L for Golden Retrievers and Miniature Schnauzers), or a high-sensitivity cardiac troponin I concentration higher than the laboratory-provided reference range (> 0.06 ng/mL) were offered participation in the longitudinal arm of the study if the owner was willing to change the diet to 1 of 6 extruded, GI, pulse-free, intervention diets. Serum was collected from these dogs 12 months after the diet change.

Statistical significance was defined as values of P < .05 and values of q (false discovery rate) < .10, which was considered to provide an acceptable degree of confidence (< 10% chance) that there were no false discoveries. This estimate of the false discovery rate (q value) was calculated to account for multiple comparisons inherent to metabolomic-based studies. Random forest analysis was used to define metabolites that contributed the most to group binning when comparing groups (irrespective of statistical significance), and these were displayed as biochemical importance plots.

Results

For the present study, baseline serum samples from 23 dogs eating GF diets (7 Doberman Pinschers, 8 Golden Retrievers, and 8 Miniature Schnauzers) and 79 dogs eating GI diets (30 Doberman Pinschers, 35 Golden Retrievers, and 14 Miniature Schnauzers) were analyzed (Figure 1). In addition, blood samples obtained 12 months after changing to a GI, pulse-free, intervention diet were available for 17 dogs (8 that were originally eating a GF diet and 9 that were originally eating a GI diet). Clinical details of these dogs and the diets have been previously reported.

Untargeted global metabolomic analysis detected 881 metabolites in these dogs, of which 832 were unnamed (ie, had an unknown structural identity) and 49 were named (ie, had a known structural identity).
Principal component analysis did not reveal high-level diet group differences but did show some separation by breed (Supplementary Figure S1). Breed distribution was not significantly ($P = .22$) different between the GF and GI groups.

**Diet group differences at baseline**

There were 46 metabolites that were significantly higher and 82 metabolites that were significantly lower in the GF group than the GI group (Table 1). Notable metabolite differences between diet groups were found for some biochemical pathways. Urea cycle intermediates (eg, homoarginine, N-acetylcarbamic acid, N-delta-acetylornithine, and N2,N5-diacetylornithine) were higher in the GF group than the GI group. Metabolites related to glutathione synthesis and turnover (eg, cysteine, cysteine sulfinic acid, and gamma-glutamylglutamine) were lower in the GF group than the GI group. Succinylcarnitine (C4-DC), which is related to glycolysis and energy production, was lower in the GF group than the GI group. Some metabolites related to phospholipid metabolism (eg, 1-palmitoyl-2-palmitoleoyl-GPC [16:0/16:1], 1-palmitoyl-2-oleoyl-GPC [16:0/18:1], 1-palmitoyl-2-arachidonoyl-GPC [16:0/20:4n6], and 1-palmitoyl-2-oleoyl-GPE [16:0/18:1]) were lower in the GF group than the GI group, whereas 1-linoleoyl-2-linolenoyl-GPC (18:2/18:3) was significantly higher in the GF group than the GI group. Microbiome-associated metabolites related to aromatic amino acids (eg, kynurenate and anthranilate) were significantly lower in the GF group than the GI group. Several vitamin A and B6 metabolites such as retinol (vitamin A), carotene diol (2), and pyridoxal were significantly lower in the GF group than the GI group. Metabolites related to pyrimidine nucleotides (eg, pseudouridine, cytidine, and 2'-deoxyuridylic acid) were significantly lower in the GF group than the GI group. Notably, taurine was not significantly different between diet groups, and only 2 of 23 metabolites related to taurine pathways (cystathionine and cysteine sulfenic acid) were different between groups (ie, significantly higher in the GF group than the GI group).
Table 1—Metabolites that were significantly different between diet groups at baseline, as determined by untargeted global metabolomic profiling, for 23 dogs eating grain-free (GF) diets and 79 dogs eating grain-inclusive (GI) diets.

| Metabolite                                      | Fold change | P value  | q value  |
|------------------------------------------------|-------------|----------|----------|
| X-25419                                        | 6.65        | < .0001  | < .0001  |
| Trigonelline (N’-methylnicotinate)              | 2.91        | < .0001  | < .0001  |
| Homoarginine                                    | 3.68        | < .0001  | < .0001  |
| X-26008                                        | 9.60        | < .0001  | .0001    |
| Tryptophan betaine                              | 14.86       | < .0001  | < .0001  |
| 1-Linolenoyl-GPC (18:3)                         | 1.86        | < .0001  | .0004    |
| Maltol sulfate                                  | 3.40        | < .0001  | .0004    |
| 1-Linoleoyl-2-linolenoyl-GPC (18:2/18:3)       | 1.90        | .0001    | .0038    |
| 1-Palmityloleyl-2-linolenoyl-GPC (16:1/18:3)   | 1.60        | .0001    | .0041    |
| N2,NS-diacylornithine                          | 5.25        | .0001    | .0054    |
| Arginine                                       | 8.86        | .0002    | .0070    |
| Cysteine sulfonic acid                         | 1.25        | .0002    | .0072    |
| N-delta-acetylorothione                         | 5.02        | .0003    | .0085    |
| 1-Lignoceryl-GPC (24:0)                         | 1.33        | .0003    | .0085    |
| X-11795                                        | 1.35        | .0003    | .0085    |
| 1-Steroyl-2-oleoyl-GPI (18:0/18:1)             | 1.27        | .0006    | .0129    |
| Valylglycine                                    | 3.28        | .0008    | .0157    |
| 5-HEPE                                         | 1.50        | .0018    | .0250    |
| Ethyl beta-glucopyranoside                      | 2.18        | .0022    | .0286    |
| Linolenate [alpha or gamma; (18:3n3 or 6)]     | 1.93        | .0032    | .0356    |
| N-acetylasparagine                              | 1.22        | .0035    | .0388    |
| Quinate                                        | 15.47       | .0050    | .0464    |
| 1-Oleoyl-GPI (18:1)                             | 1.41        | .0054    | .0482    |
| Linolenoylcarnitine (C18:3)                     | 1.35        | .0074    | .0579    |
| Eicosenoate (20:1)                              | 1.43        | .0082    | .0611    |
| 18-Methylnonadecanoate (20:0)                   | 1.27        | .0101    | .0679    |
| 1-Stearoyl-2-linoleoyl-GPI (18:0/18:2)         | 1.16        | .0105    | .0680    |
| 2’-Deoxyuridine                                 | 1.15        | .0108    | .0680    |
| 1-Oleoyl-GPC (18:1)                             | 1.15        | .0111    | .0693    |
| Cis-3,4-methyleneheptanoylcarnitine             | 2.33        | .0116    | .0706    |
| 1-Eicosapentaenoylglycerol (20:5)               | 1.27        | .0116    | .0706    |
| Indoleacrylate                                  | 2.47        | .0128    | .0751    |
| 1-Oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)     | 1.31        | .0132    | .0751    |
| 2-Oxogarginine                                  | 1.52        | .0153    | .0813    |
| Solanidine                                      | 6.43        | .0158    | .0821    |
| Imidazole lactate                               | 1.22        | .0167    | .0871    |
| Methylphosphoryl sulfate (2)                    | 1.74        | .0173    | .0874    |
| Arachidate (20:0)                               | 1.33        | .0181    | .0874    |
| Gamma-tocopherol/beta-tocopherol               | 3.12        | .0184    | .0874    |
| N-acetylcitrulline                              | 1.77        | .0186    | .0874    |
| Glyceroophosphinositol                          | 1.15        | .0189    | .0875    |
| X-11478                                        | 4.98        | .0191    | .0875    |
| Cystathionine                                   | 1.38        | .0217    | .0955    |
| Glycerol 3-phosphate                            | 1.34        | .0251    | .0993    |
| 1-Linolenoylglycerol (18:3)                     | 1.82        | .0254    | .0994    |
| Gamma-CEHC sulfate                              | 1.51        | .0266    | .0996    |
| N-nonemethylynarginine                         | 0.68        | < .0001  | < .0001  |
| 2’-Oxindole-3-acetate                           | 0.37        | < .0001  | .0001    |
| 2’-Deoxycytidine                                | 0.71        | < .0001  | .0004    |
| Cytidine                                       | 0.72        | .0001    | .0031    |
| Pyridoxal                                       | 0.61        | .0002    | .0054    |
| 1-Palmityloyl-GPE (16:0)                        | 0.77        | .0002    | .0061    |
| X-25371                                        | 0.71        | .0003    | .0085    |
| 4-Vinylphenol sulfate                           | 0.41        | .0004    | .0089    |
| Pyridoxate                                      | 0.53        | .0005    | .0104    |
| Gamma-glutamylvaline                            | 0.64        | .0006    | .0121    |
| Sphingomyelin (d18:0/18:0, d19:0/17:0)           | 0.63        | .0009    | .0169    |
| 3,5-Dichloro-2,6-dihydroxybenzoic acid          | 0.44        | .0010    | .0182    |
| Kynurenate                                      | 0.52        | .0012    | .0212    |
| X-25247                                        | 0.27        | .0012    | .0213    |
| Gamma-glutamylycine                             | 0.79        | .0014    | .0228    |
| N-acetylaniline                                 | 0.88        | .0015    | .0244    |
| Gamma-glutamylyglycine                          | 0.81        | .0016    | .0244    |
| Anthranilate                                    | 0.69        | .0017    | .0244    |
| Gamma-glutamylytyrosine                         | 0.73        | .0017    | .0244    |
Table 1—(continued).

| Metabolite                                                   | Fold change | P value | q value |
|--------------------------------------------------------------|-------------|---------|---------|
| Retinal                                                      | 0.70        | .0017   | .0244   |
| 1,5-Anhydroglucitol (1,5-AG)                                 | 0.72        | .0018   | .0250   |
| Succinylcarbinine (C4-DC)                                    | 0.74        | .0021   | .0286   |
| 1-Palmitoyl-2-oleoyl-GPC (16:0/18:1)                         | 0.89        | .0023   | .0289   |
| 4-Vinylguaiacol sulfate                                      | 0.10        | .0025   | .0310   |
| Glycine                                                      | 0.85        | .0026   | .0318   |
| Betaine                                                      | 0.84        | .0021   | .0356   |
| Leucine                                                      | 0.88        | .0031   | .0356   |
| Gamma-glutamylglutamine                                     | 0.87        | .0032   | .0356   |
| Palmitoylcarbinine (C16)                                     | 0.76        | .0040   | .0433   |
| Stachydrine                                                  | 0.49        | .0042   | .0440   |
| 2,6-Dihydroxybenzoic acid                                   | 0.56        | .0043   | .0447   |
| N1-methyl-2-pyridone-5-carboxamide                           | 0.58        | .0049   | .0464   |
| X-13695                                                     | 0.51        | .0049   | .0464   |
| Tyrosine                                                     | 0.85        | .0050   | .0464   |
| N-stearoyl-sphinganine (d18:0/18:0)                          | 0.63        | .0050   | .0464   |
| Retinol (vitamin A)                                          | 0.78        | .0050   | .0464   |
| 5,6-Dihydouridine                                            | 0.85        | .0053   | .0479   |
| 1-Palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)                 | 0.86        | .0056   | .0489   |
| Cysteinylglycine                                             | 0.75        | .0058   | .0493   |
| Pseudouridine                                                | 0.88        | .0058   | .0493   |
| 3-Bromo-5-chloro-2,6-dihydroxybenzoic acid                   | 0.48        | .0060   | .0499   |
| 1-Palmitoyl-2-linoleoyl-GPC (16:0/18:2)                      | 0.88        | .0068   | .0562   |
| Palmitoyl dihydrosphingomyelin (d18:0/16:0)                  | 0.84        | .0070   | .0569   |
| 1-Methylnicotinamide                                         | 0.57        | .0072   | .0573   |
| N2,N2-dimethylguanosine                                      | 0.88        | .0075   | .0579   |
| Carotene diol (2)                                            | 0.49        | .0076   | .0579   |
| 1-Methylhistamine                                            | 0.68        | .0081   | .0617   |
| Proline                                                      | 0.85        | .0084   | .0617   |
| 1-(1-Enyl-palmitoyl)-GPE (P-16:0)                            | 0.77        | .0085   | .0619   |
| N-acetylglucosamine/N-acetylgalactosamine                    | 0.82        | .0088   | .0627   |
| 5-Dodecenoylcarnitine (C12:1)                                | 0.68        | .0098   | .0679   |
| 1-(1-Enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)        | 0.76        | .0098   | .0679   |
| Alpha-CEHC glucuronide                                       | 0.63        | .0100   | .0679   |
| Sphingosine 1-phosphate                                      | 0.85        | .0107   | .0680   |
| X-25422                                                     | 0.80        | .0107   | .0680   |
| 3-Decenoicarnitine                                           | 0.55        | .0108   | .0680   |
| Cys-gly, oxidized                                           | 0.73        | .0113   | .0698   |
| 1-(1-Enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)            | 0.80        | .0122   | .0736   |
| 2-Aminophenol sulfate                                       | 0.55        | .0124   | .0736   |
| X-11843                                                     | 0.38        | .0131   | .0751   |
| 1-Palmitoyl-2-linoleoyl-GPE (16:0/18:2)                      | 0.75        | .0132   | .0751   |
| Dodecanedioate (C12-DC)                                     | 0.25        | .0133   | .0751   |
| Nicotinamide                                                 | 0.36        | .0134   | .0751   |
| 1,2-Dipalmitoyl-GPC (16:0/16:0)                              | 0.86        | .0138   | .0761   |
| Glycosyl-N-stearoyl-sphingosine (d18:1/18:0)                 | 0.79        | .0143   | .0783   |
| 5-Methylnorleucine                                           | 0.70        | .0145   | .0788   |
| Ethylmalonate                                                | 0.79        | .0148   | .0792   |
| [N(1) + N(8)]-acetylperimidine                               | 0.72        | .0173   | .0874   |
| 1-Palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)                   | 0.83        | .0174   | .0874   |
| 1-(1-Enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)        | 0.81        | .0176   | .0874   |
| N-palmitoylglucose                                           | 0.69        | .0177   | .0874   |
| Gamma-glutamylphenylalanine                                  | 0.87        | .0183   | .0874   |
| X-12411                                                     | 0.51        | .0185   | .0874   |
| N-palmitoyltaurine                                           | 0.57        | .0186   | .0874   |
| 1-Stearoyl-GPE (18:0)                                        | 0.89        | .0190   | .0875   |
| 1-Palmitoyl-2-linoleoyl-GPA (16:0/18:2)                      | 0.76        | .0204   | .0925   |
| Phenol glucuronide                                           | 0.56        | .0207   | .0931   |
| 1-Palmitoyl-2-oleoyl-GPE (16:0/18:1)                         | 0.80        | .0213   | .0949   |
| Guanidinoacetate                                             | 0.80        | .0214   | .0949   |
| Octadecanadioycarnitine (C18-DC)                             | 0.82        | .0228   | .0992   |
| Alanine                                                      | 0.87        | .0251   | .0993   |
| Hexadecahydroximate                                          | 0.73        | .0258   | .0996   |

Metabolites with a fold change > 1.0 were higher in the GF group than in the GI group, and metabolites with a fold change < 1.0 were lower in the GF group than in the GI group. The q value represents the false discovery rate.
Random forest analysis computed the top 30 metabolites that differentiated the GF and GI groups at baseline with 93% predictive accuracy (Figure 2). Most of these metabolites suggested key differences in lipid metabolism (9 metabolites) and amino acid metabolism (7 metabolites). The top differentiating metabolite was unnamed (X-25419), and 3 other unnamed metabolites were part of this biochemical importance plot.

**Figure 2**—Biochemical importance plot, developed by means of random forest analysis, showing the top 30 metabolites that contributed most to group binning (GF or GI diet group) at baseline for 23 dogs eating GF diets and 79 dogs eating GI diets. Predictive accuracy was 93%. The super pathway for each metabolite is indicated by color.

**Table 2**—Metabolites that were significantly different between diet groups 12 months after a diet change, as determined by untargeted global metabolomic profiling, for 8 dogs eating GF diets and 9 dogs eating GI diets before the diet change.

| Metabolite                              | Fold change | P value | q value |
|-----------------------------------------|-------------|---------|---------|
| X-25419                                 | 6.07        | .0003   | .0642   |
| Myristoleate (14:1n5)                   | 2.19        | .0004   | .0642   |
| X-25420                                 | 8.62        | .0004   | .0642   |
| Palmitoleoyl carnitine (C16:1)          | 1.74        | .0012   | .0901   |
| X-25417                                 | 3.04        | .0020   | .0901   |
| S-methylcysteine sulfoxide              | 1.39        | .0026   | .0992   |
| Uridine 5'-monophosphate (UMP)          | 0.32        | .0004   | .0642   |
| 2R,3R-dihydroxybutyrate                 | 0.64        | .0008   | .0901   |
| 1-(1-Eenyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4) | 0.63 | .0014 | .0901 |
| 1,2-Dilinoleoyl-GPC (18:2/18:2)         | 0.57        | .0015   | .0901   |
| Gamma-glutamylthreonine                 | 0.64        | .0017   | .0901   |
| 12-HHTE                                 | 0.24        | .0017   | .0901   |
| X-25247                                 | 0.22        | .0017   | .0901   |
| Threonine                               | 0.63        | .0018   | .0901   |
| 4-Ethylphenylsulfate                    | 0.26        | .0019   | .0901   |
| Thromboxane B2                          | 0.24        | .0023   | .0973   |
| X-13695                                 | 0.33        | .0025   | .0992   |

**Figure 3**—Biochemical importance plots, developed by means of random forest analysis, showing the top 30 metabolites that contributed most to group binning (baseline vs 12 months after diet change) for 8 dogs with subclinical cardiac abnormalities fed a GF diet at baseline that were switched to a GI, pulse-free, intervention diet for 12 months (A) and for 9 dogs with subclinical cardiac abnormalities fed a GI diet at baseline that were switched to a GI, pulse-free, intervention diet for 12 months (B). Predictive accuracy was 87% (A) and 60% (B). The super pathway for each metabolite is indicated by color.
Effect of diet change

Comparison of diet groups at 12 months

Comparison of dogs with subclinical abnormalities in the GF group (1 Doberman Pinscher, 4 Golden Retrievers, and 3 Miniature Schnauzers) and GI group (3 Doberman Pinschers and 6 Golden Retrievers) 12 months after the diet change found 6 metabolites that were higher and 11 metabolites that were lower in the GF group than the GI group (Table 2). Only 3 of these 17 metabolites were also significantly different between diet groups at baseline, and all 3 of these were unnamed (X-25419 was higher in the GF group and X-25247 and X-13695 were lower in the GF group at both time points).

Comparison of baseline and 12 months after diet change within each diet group

No metabolites were significantly different between baseline and 12 months after diet change for the GF group or for the GI group (Supplementary Tables S1 and S2).

For dogs in the GF group, the biochemical importance plot for the within-group time point comparison showed high predictive accuracy (87%), with the top-ranking metabolites suggesting key differences 12 months after diet change related to lipid metabolism (14 metabolites), cofactors and vitamins (4 metabolites), and xenobiotics (4 metabolites; Figure 3).

For dogs in the GI group, the biochemical importance plot for the within-group time point comparison suggested key differences after diet change for metabolites related to lipid metabolism (14 metabolites) and amino acid metabolism (5 metabolites), with 60% predictive accuracy (Figure 3).

Discussion

Untargeted global metabolomic analysis of the healthy dogs in this study revealed numerous biochemical differences between dogs eating GF diets and those eating GI diets. Most of the biochemical differences between groups were related to lipid metabolism and amino acid metabolism and were statistically significant at a low false discovery rate. More granular group differences were related to pathways involving urea cycle intermediates, glutathione synthesis and turnover, phospholipid metabolism, vitamins and cofactors, and pyrimidine nucleotides. The results of this study showed some similarities to a publication that applied metabolomic profiling to food samples (foodomics).15 For example, the food-based study also found lower vitamins and cofactors and higher xenobiotics in diets associated with DCM, compared with diets not associated with DCM, which was similar to our findings for these metabolites in the blood of dogs eating GF and GI diets.15 While it is not surprising that biochemical compounds found in food would be detectable in the blood of dogs, the metabolomic profile is not expected to be identical to the foodomic profile because of changes that biochemical compounds undergo associated with absorption and metabolism.

Several unnamed compounds were also found to be biomarkers of GF diet ingestion, but their identity and clinical importance are not known. The top differentiating metabolite (X-25419, which, in the GF group, was 6.65 times that in the GI group at baseline) was reported to be higher by a factor of 7.67 fold in dog foods associated with DCM compared with dog foods that have not been associated with DCM in the previous foodomics study, suggesting that diet is the source of this metabolite in the blood of dogs in the GF group.15 This unnamed compound was also present in GI diets but to a lesser degree. A study18,19 in people identified X-25419 in blood and urine to be associated with fish and olive oil ingestion, and a study20 of dogs with mitral valve disease found that X-25419 was significantly different between healthy dogs predisposed to heart disease and dogs with mild heart disease. Fish is a common protein source for many types of dog diets, but diet was not reported in the dog study.18 Until structural elucidation is performed, speculation about the clinical importance of X-25419 is not possible.

The present study cannot be used to determine whether any of these biochemical differences (involving named or unnamed metabolites) predispose some dogs that eat GF diets to cardiac abnormalities, but it demonstrated that ingestion of GF diets affects the biochemical footprint of dogs. Therefore, the impact of diet should be considered in the design and analysis of future studies evaluating this clinical problem and of metabolomic studies in companion animals in general. Some of the super pathways that were different between diet groups in this study might be a basis for hypothesis generation in future studies.

We evaluated the effect of transition to an intervention diet in a subset of dogs found to have subclinical cardiac abnormalities by looking for between-group differences at baseline and again 12 months after a diet change and by looking at within-group time point comparisons (ie, baseline vs 12 months after diet change for each group). Fewer diet group biochemical differences were found between the GF and GI groups 12 months after this subgroup of dogs with subclinical cardiac abnormalities transitioned from their original diet to an intervention diet. Most metabolite differences between groups at baseline were no longer found 12 months after diet change, with the exception of 3 unnamed metabolites, which remained significantly different (2 remained lower and 1 remained higher in the GF group). The clinical importance of these unnamed compounds for which group differences persisted cannot be determined until the compounds are structurally elucidated. The loss of most metabolomic group distinctions at 12 months was likely attributable to the dietary unification provided by the intervention diets; however, the clinical importance of these changes is unknown. Some of these changes, such as higher vitamin and cofactor metabolites after diet change, might benefit cardiac health, but this requires further research. The finding of new metabolites that differentiated diet groups at 12 months but had not differentiated
diet groups at baseline might be a result of the com-
parison involving a smaller group of dogs with sub-
clinical cardiac abnormalities at the 12-month time
point, compared with larger group of dogs (most of
which did not have subclinical cardiac abnormalities)
at baseline. Therefore, this comparison might reflect
metabolomic differences between these 2 groups
that were unrelated to diet or due to other factors
that could have varied between groups (eg, environ-
mental, genetic, microbiomic, and residual cardiac
abnormalities).

When within-group comparisons of dogs with
subclinical cardiac abnormalities were performed
for each individual diet group before and 12 months
after a diet change, no metabolite differences were
statistically significant. Low statistical power could
have impacted these results, because a relatively
small number of dogs in each group were assessed
before and after diet change (8 dogs in the GF group
and 9 dogs in the GI group). Random forest analy-
sis, which ranks the top metabolites contributing
to group differences regardless of statistical signifi-
cance, showed a higher degree of predictive accu-
rate for these metabolites before and after a diet
change for the GF group (87%) than for the GI group
(60%). Fewer changes for dogs in the GI group after
diet change could be explained by the fact that the
intervention diets were similar to the GI diets in their
gra in inclusivity and pulse content. Key differences
after the diet change for the GF group were related
to lipid metabolism, cofactors and vitamins, and
xenobiotics. These changes were probably largely
due to the diet change, but their clinical impor-
tance is uncertain. The relationship between these
metabolomic changes and the potential for disease
predisposition or resolution cannot be determined
from this study, but these findings can provide some
hypotheses to drive future research.

A strength of the present study was the absence
of advanced cardiac disease, which can change
the metabolome. However, it is important to recog-
nize that this methodology is only a biochemical
snapshot in time. There are several limitations
that should be considered for study interpretation.
The diet groups were relatively unbalanced, with
more dogs in the GI group, because they reflected
the population of dogs presenting for study enroll-
ment. The time point comparisons might have suf-
fered from low power and breed imbalance because
of the small number of overtly healthy dogs that
were found to have subclinical cardiac abnormali-
ties and underwent a diet change with subsequent
reanalysis of metabolomics. The complex interplay
do diet and health is not fully understood, and there
could be other modifying influences that deter-
mine the effect of diet on the metabolome, includ-
ing the microbiome, environmental influences, and
behavioral influences. Some of these factors could also
lead to changes in gene expression, which could
affect the metabolites detected in the blood. The
dogs in the present study were overtly healthy,
but we did not account for environmental fac-
tors. High-level analysis with principal component
analysis suggested some metabolomic profile sep-
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Supplementary Materials

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