Targeted metabolomics with Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) highlights metabolic differences in healthy and atopic Staffordshire Bull Terriers fed two different diets, a pilot study

Master's thesis
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Tiivistelmä – Referat – Abstract

While anecdotal evidence has long claimed that a raw meat–based diet (RMBD) improves the metabolic health of canines, no rigorous scientific study has clarified this issue. Canine atopic dermatitis (CAD) has also been linked to metabolic health, but its relation to diet remains poorly understood. This study investigates whether dietary choice is linked to metabolic health in healthy and CAD-diagnosed canines via targeted serum and urine metabolomic analysis of polar, non-ionic metabolites, as well as whether the underlying CAD condition modulates the response to nutritional intake.

Serum metabolites of client-owned Staffordshire Bull Terriers, divided into CAD-diagnosed (n=14) and healthy (n=6) cohorts, were studied. Urine metabolites of a subset of the CAD-diagnosed canines (n=8) were also studied. The canines were split into two cohorts based on diet. The first cohort were fed a commercially available high-fat, moderate-protein, low-carbohydrate RMBD (n=11, CAD diagnosed n=8, healthy n=3). The second cohort were fed a commercially available moderate-fat, moderate-protein, high-carbohydrate kibble diet (KD) (n=9, CAD diagnosed n=6, healthy n=3). The diet intervention period lasted approximately 4.5 months (median 135d). Statistical analysis of the serum profiles across all dogs (n=20) and the urine profiles of the CAD-diagnosed subset (n=8) were performed.

The KD cohort was found to have higher concentrations of methionine than the RMBD cohort, both in serum (all dogs, $p<0.0001$) and in urine (CAD-only cohort, $p<0.0002$), as well as cystathionine and 4-pyridoxic acid. Methionine plays important roles in homocysteine metabolism, and elevated levels have been implicated in various pathologies. The CAD (n=14) cohort dogs showed starker metabolic changes in response to diet regarding these pathways compared to the healthy (n=6) cohort. However, there was no significant change in CAD severity as a result of either diet. Likely due to the higher meat content of the RMBD, higher concentrations of several carnitines and creatine were found in the RMBD cohort. Citrulline was found in higher concentrations in the KD cohort. While the findings from this experiment provide insight into the relationship between diet and the serum and urine metabolite profiles of canines, they also suggest that neither diet significantly affected CAD severity.

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raw meat-based diet, targeted metabolomics, canine atopic dermatitis, kibble diet, diet intervention

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Tiivistelmä – Referat – Abstract

Anekdotiskt bevis har länge påstått att en rå köttbaserad diet (RKBD) förbättrar hundens metaboliska hälsa. Dock har ingen noggrann vetenskaplig studie klargjort denna fråga. Hund atopisk dermatit (HAD) har också kopplats till metabolisk hälsa, men dess förhållande till diet har ej studerats via studien av hundens metabolism tidigare. Denna studie undersöker om diet val är kopplade till metabolisk hälsa hos friska och HAD-diagnostiserade hundar genom att köra riktad blodserum- och urinmetabolomik analys av polära, icke-joniska metaboliter, samt för att svara huruvida det underliggande HAD-sjukdomen modulerar hur hunden reagerar till dess diet.

Serum metaboliter från Staffordshire bullterrierrar som indelades till antingen HAD-diagnoserade (n=14) eller friska (n=6) studerades. Urinmetaboliter från en portion av de HAD-diagnoserade hundarna (n=8) studerades också. Hundarna indelades vidare till två dietgrupper. Förstå gruppen åt en kommersiell RKBD som hade låg kolhydrathat och högt protein samt fetthalt (n=11, HAD diagnoserade n=8, friska n=3). Andra gruppen (n=9, HAD diagnoserade n=6, friska n=3) åt ett kommersiellt torrfoder (TF) som hade hög kolhydrathalt, måttlig proteinhalt samt låg fetthalt. Dietinterventionen räckte cirka 4.5 månader (median=135d). Statistisk analys of hundarnas serum metabolitprofiler (n=20) samt urin metabolitprofiler från en del av HAD-diagnoserade hundarna (n=8) kördes och resultaten studerades. Efter dietinterventionen hade gruppen som åt TF dieten betydligt högre koncentrationer av metionin än RKBD gruppen, både i blodserum (alla hundar, n=9, $p<0.0001$) och i deras urin (HAD-gruppen, n=6, $p<0.0002$). I urinet fanns det också betydligt högre koncentrationer cystationin samt 4-pyridoxalsyra. Dess metaboliter spelar viktiga roller inom metaboliska rutten som bryter ner homocystein, där speciellt förhöjda metioninkoncentrationer har visats sig korrelera med diverse kroniska sjukdomar. Större ändringar i metabolitkoncentrationerna som påföljd av diet observerades i HAD-gruppen (n=14) jämfört med friska gruppen (n=6). Dock fanns det ingen betydlig ändring på deras HAD-symtom som påföljd av diet efter dietinterventionen avslutades. Högre hater av karnitiner samt kreatin hittades i RKBD-gruppens blodserum, antagligen på grund av höga kötthaltet i deras diet. Betydligt högre citrullinhalter hittades också i blodserumet från TF-gruppen. Resultaten från detta experiment ger insikt till hur diet och hundens blodserum samt urin metabolitprofiler påverkar varandra. Vidare visar resultatet att varken TF eller RKBD dieten betydligt ändrar på HAD-symtom.

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rå köttbaserad diet, riktad metabolomik, hundatopisk dermatit, torrfoder, dietintervention

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Foreword and Acknowledgments

I would like to thank Dr. Anna Hielm-Björkman for providing me the opportunity to work with her and the DogRisk team. When beginning this project in the fall of 2016, I had no idea how immersive the topic would turn out to be. I did not imagine that researching the world of nutrition and metabolomics for this project would have me traveling around the world several times, as well as completely transform my relationship to food, stress, and well-being. Working on this project has allowed me to become familiar with the role of researcher, as well as the complex world of metabolism and both our dogs' and our own relation to food. I would also like to express my gratitude to the Finnish Institute for Molecular Medicine (FIMM) Metabolomics Centre for running the metabolomic analysis, as well as providing helpful insight and guidance for the statistical analysis. I have presented portions of this work, as well as this work in its entirety as posters at several conferences, including the 2017 Metabolic Therapeutics Conference (Tampa, FL, USA), the 66th annual American Society for Mass Spectrometry Conference 2018 (San Diego, CA, USA), the 14th International Conference of the Metabolomics Society 2018 (Seattle, WA, USA), the 2nd Helsinki Evidence-based Raw Food seminar (Helsinki, Finland) 2018, the 1st Nordic Metabolomics Society Conference (Örebro, Sweden) 2018, Clinical Metabolomics Workshop Copenhagen 2018 (Copenhagen, Denmark), Metabolic Health Summit 2019 (Long Beach, CA, USA), Gordon Research Conference: Human Metabolomics 2019 (Ventura, CA, USA), Nordic Metabolomics Society Workshop and Throne Holst Symposium 2019 (Oslo, Norway). During the completion of this master's thesis, I published this experiment as an article.[1]

Abbreviations

RMBD	Raw meat based diet
KD	Kibble diet
CAD	Canine atopic dermatitis
CADESI	Canine Atopic Dermatitis Extent and Severity Index
AD	Atopic dermatitis
MS	Mass spectrometer/spectrometry
MS/MS	Tandem mass spectrometer
GC-MS	Gas-chromatography
LC-MS	Liquid-chromatography mass spectrometry
RP-LC	Reversephase liquid-chromatography
ESI	Electrospray ionization
HILIC	Hydrophilic intreraction liquid-chromatography
QqQ	Triple quadrupole
CID	Collision induced dissociation
SRM	Selected reaction monitoring
MRM	Multiple reaction monitoring
UPLC-MS	Ultra-performance liquid-chromatography
IS	Internal/istopically labeled Standard
MetS	Metabolic syndrome
GLM	General linear model
ANOVA	Analysis of Variance

FDR	False discovery rate
LSD	Least squares discriminant analysis
VIP	Variable importance in projection
PFC	Protein:fat:carbohydrate (ratio)
%ME	(percent) Metabolizable energy
PLS-DA	Partial least squares discriminant analysis
LOOCV	Leave-one-out cross validation
MCT	Medium-chain triglyceride
FIMM	Finnish Institute of Molecular Medicine
SD	Standard deviation
B12	Vitamin B_{12}
ELLA	Animal Experiment Board in Finland

1 Introduction

This master's thesis work was performed the University of Helsinki Small animal and Equine Hospital, and Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine at the University of Helsinki, and is the metabolomics portion of a 'nutri-omics' research project, which was initiated in 2013 by the DogRisk research group led by Dr. Anna Hielm-Björkman.[2,3]

Improving the length of pet healthspan remains a long-term goal in research of the health-nutrition axis. To achieve this, most research focuses on practical solutions, for example improving diet to treat chronic disease in canines.[4] To date however, little consideration of diet as a means for disease prevention has been reported in the literature.[5] It has been well established that a healthy diet in humans contributes to an increased healthspan, and that an unhealthy diet increases the risk of many pathologies.[6-8] In canines, studies to see whether certain diets help treat chronic diseases have mainly involved observing whether certain types of diets and functional foods appear to have a protective or therapeutic effect against chronic ailments.[9-11]

With the recent advancements in the field of metabolomics, it has become easier to study the relationship between an individual's metabolome and environmental factors.[12] The nascent field of canine nutritional metabolomics holds potential for both improving our understanding of canine disease risk factors and the underlying causes behind those risks.[13] However, to our knowledge using a targeted metabolomics approach to study the interactions between chronic disease states and long-term dietary interventions on canines had not previously been performed. Although kibble and raw food diets are the two predominant forms of dog feeding throughout most of the world, only a few studies exist that have compared the two. Furthermore, to date, no studies have been published that use a targeted metabolomics approach to study the effects of feeding these diets on canine health. Through the use of combined media (blood serum and urine) in the present study, I examine the extent to which the homeostasis of quantified blood metabolites are maintained, and their relationship with food intake. By using a targeted metabolomics approach, data was generated and used to determine whether, and to what extent differences in the canine blood serum and urine metabolome could be seen as a result of the two different diets, as well as between healthy and atopic individuals. I studied the metabolic relationships between diet and the chronic skin disease canine atopic dermatitis (CAD) as well as canine metabolic health in general through comparing our results with previously performed studies. This was performed by applying statistical tools and data processing protocols as described in the

literature, and then interpreting the biological implications to the best of my ability using recent relevant research. This study aims to determine the validity and practicality of using this method for providing context-inclusive answers for research questions that focus on the intersection between nutritional intervention and long-term health outcome in canines. This preliminary study began with the hypotheses that dietary choice is linked to both metabolic health as well as CAD severity in the CAD-diagnosed canines, and that a preliminary indication of whether this is the case can be seen in the targeted serum and urine metabolite profiles of polar, non-ionic metabolites. I further hypothesized that the healthy and CAD-diagnosed canines would respond differently to nutritional intake by means observable in the targeted serum and urine metabolite profiles of polar, non-ionic metabolites. Clinical metabolomics-based experiments are inherently hypothesis-generating and are hence an ideal approach for preliminary or pilot studies. Hence, through analyzing metabolites which represent a diverse yet sparse selection of metabolites simultaneously, I also aimed to generate new information that could help guide the formulation of future, more precise hypotheses. Next, I outline an overview of the literature that has forged the path to where we now are in the realm of clinical metabolomics, specifically with regards to canines and the study of underlying chronic disease states, in particular canine atopic dermatitis.

2 Literature review

2.1 Metabolites

Metabolites are a diverse class of mainly organic compounds, typically described as being any compound up to 1500 Da in size,[14] which are produced by organisms as a result of cellular metabolism.[15] In animals, the term 'metabolites' is broad and typically refers to lipids, amino acids and small peptides, nucleic acids, carbohydrates and organic acids, thiols as well as conjugates of the above-mentioned compound classes.[16] Together, they make up the organism's metabolome, also termed metabolic profile, and the term's use is almost invariably biofluid-, cell-, or biotissue-specific.[17] For example, the blood serum and urine metabolome is of great interest to health researchers.[18] The metabolome is made of compounds that are either exogenous, i.e. compounds which the organism has acquired from its environment, or endogenous, i.e. compounds which the organism itself has created via either anabolic or catabolic processes. Regardless of where they came from, metabolites are

regarded as compounds involved with the organism's metabolic processes.[19] They are the direct link between an organism's genetic expression, and its interaction with its environment, and hence the metabolic profile of an individual has been referred to as describing a biologically 'functional phenotype' (*Figure 1*).[20,21] In other words, the organism expresses genes, with which it creates proteins and enzymes in order to manipulate its metabolic profile in response to it environment.[22]

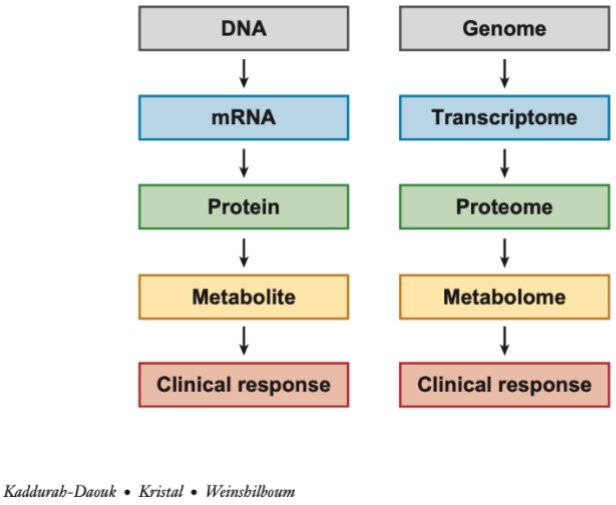

Figure 1. Overview of the dichotomy between traditional clinical biochemistry experiments (left) and its 'omics' counterpart that has become increasingly popular in clinical chemistry research. Figure adapted from illustration by Kaddurah-Daouk et al. (2008).[15]

2.2 Metabolomics

The study of whole sets of metabolites are generally referred to as metabolomics, metabonomics, or metabolic profiling.[23] The terms all refer to the detection and measurement of the metabolome of an organism's biofluids or any other biomaterial, including solids and gases.[15] The study of sets of metabolites began long before the coining of the term 'metabolomics'[24] and the field is built upon many decades of research that primarily focused on single metabolic pathways,[19] or types of compounds e.g., amino acids.[19,25] However, metabolomics specifically aims to address how metabolic flux can be observed in response to an external or internal factor across multiple, or even all metabolic activities present within a cell, tissue, biofluid or organism.[26] Although the term metabolomics is ubiquitously used in current metabolite-based studies, many distinct types of metabolomics-based analyses exist, presenting a persistent challenge when tasked with defining what metabolomics precisely describes.[24,27] Essentially, all forms of metabolomics-type analyses combine preparing and feeding a

biological sample to a highly sensitive instrument, and then interpreting the instruments output with robust statistical data processing software.[27] Due to the highly variable nature of the metabolome's physicochemical properties, as well as variability of relative metabolite concentrations, it has proved challenging to create an experimental procedure that would allow for the detection and characterization of all metabolites present in a biological sample, even when multiple instrument platforms are combined.[21] As a result, to our best knowledge no current methodology allows for a truly all-inclusive quantitative metabolome analysis and consequently certain trade-offs have to be taken into account when choosing which platform or combination of instruments to use for metabolomics-based experiments.[28] The most common instruments in use today for metabolomic analyses are nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).[29] When compared to NMR, GC-MS and LC-MS are considerably more sensitive instruments and hence allow lower level of detection for metabolites within samples. As such LC-MS platforms have become the more popular of the two options with regards to health research, where generally many biofluid metabolites are found in relatively small concentrations which in many cases do not need to vary much to illicit large-scale phenotypic changes.[30] Upon detection, the measurement of metabolites and other small-molecule concentrations within the sample can be either qualitative or quantitative depending on the type of experimental setup, which in turn is dependent on the aim of the experiment.[21] When using LC-MS, metabolomics experiments that yield qualitative data regarding the sample's metabolome are generally referred to as untargeted metabolomics, and metabolomics experiments that yield quantitative data regarding a defined set of metabolites present in the sample's metabolome are generally referred to as targeted metabolomics.[21] Here, we focus on targeted metabolomics using an ultra-performance liquid chromatography-tandem triple quadrupole mass spectrometry platform (UPLC-MS/MS), a set of techniques which may enable quantitative metabolite measurements in canine urine and blood serum samples in a clinical chemistry setting.

2.3 Clinical targeted metabolomics experiments

Targeted metabolomics analysis is most often performed with a liquid chromatography tandem mass spectrometry (LC-MS/MS) instrument, which has become the gold standard in clinical settings.[31] Here are the key considerations required at each step of the workflow for the successful analysis of non-

ionic, polar metabolites in blood serum and urine samples by using targeted metabolomics with the LC-MS/MS-based approach in a clinical setting as has been recommended in the literature.[21,30,32,33]

2.3.1 Experiment design

Designing an experiment properly is perhaps the most important step to performing a successful experiment. A failure in design may make all subsequent steps unusable, or biologically irrelevant.[34] The design must hence adequately address the aims of the experiment, which in turn is to answer the experiment's hypotheses. In a clinical research setting, the use of metabolomics is most often aimed to complement more traditional clinical chemistry experiments, where metabolomics-based experiments may be used as a 'first-pass' approach, generally thought of as a means to generate novel hypotheses. The data offers a broad view of metabolic processes which may then be used to better pinpoint where exactly within the metabolome, or within a certain set of metabolic processes it would be wise to follow up with more precise clinical chemistry assays.[33] Several key considerations need to be determined. First, it is necessary to determine what data would be required in order for the research hypothesis to be answerable. This includes choosing which bio-samples should be collected for analysis, which analytical method should be chosen to analyze the collected samples, and which statistical methods should be used for subsequent data analysis. Second, the minimum cohort size necessary for the results of a tested variable to be considered statistically significant in metabolomics-based studies needs to be determined. Often a pilot study with a small sample size is used in order to give an indication of whether the research question should be further studied using a larger cohort, which would allow for a more precisely defined hypothesis.[35] Clinical pilot studies on the other hand often rely on the minimum case-control inclusion requirements which would allow for subsequent power calculations for larger-scale studies to be considered reliable.[36] Lastly, clinical research also requires approval from an ethics committee.[37]

2.3.2 Sample collection

When the samples are collected, handled and stored, the aim is to do so in a uniform manner which allows for inter and intra-individual sample variability to be kept to a minimum, as well as in a way that allows for the sample's metabolite concentrations to remain stable.[38] This is achieved by controlling for both the conditions under which the sample is collected, as well as the conditions that the collected sample is then stored in prior to preparation for LC. Certain criteria apply to both the

collection of blood and urine samples, namely the samples should all be taken at approximately the same time of day to prevent metabolic flux due to the individuals' circadian rythym[39] and fasted samples should always be collected to prevent temporary flux due to meals.[40] Furthermore, for blood serum samples, it is essential that the locus of sample collection is the same for all individuals and for urine samples it is recommended that they are collected mid-stream.[41] Blood serum samples are allowed to clot and the resulting biomaterial, including cells and debris is removed with centrifugation. Both blood serum and urine samples should be stored in -80°C after collection, and freeze-thaw cycles should be kept to a minimum.[33,42]

2.3.3 Sample preparation

After the blood serum or urine sample has been collected and stored, several preparatory steps must be taken for the successful analyses of the targeted metabolites prior to chromatographic analyte separation and subsequent analysis with the MS/MS instrument.21 First, a precise amount of each sample destined for analysis is aliquoted and a precise amount of isotopically labeled standards (IS) for each of the targeted metabolites are added directly to it as well as to replicates of the sample, which are included to further increase the reliability of the analysis. Besides allowing for absolute quantification of the metabolite concentration in the sample, the IS can also allow for different sample batches, i.e. samples that are analyzed during different 'runs' to be compared.21 Second, a sample clean-up step called metabolite extraction is typically performed. In targeted metabolomics, the aim is primarily to extract the metabolites of interest as effectively as possible while also aiming to remove as many compounds as possible from the sample that are not of interest.33 This is best achieved by taking advantage of the common physicochemical properties of the targeted metabolites, such as solvent polarity, pH and temperature. Blood serum samples are first treated with a protein precipitation step, where typically an organic solvent, e.g. methanol or acetone is added, and the precipitate is removed via filtration.43 Due to their low protein concentration, urine samples typically do not require this step and instead preparation focuses on the interaction that the sample will have with the column it will subsequently be run through. For example, a large ratio of organic solvent is added to urine samples destined for separation with HILIC columns, as the high water content of urine hinders the HILIC column from separating the analytes smoothly.30 For both blood serum and urine samples, a solid-phase extraction (SPE) is typically employed, where the metabolites of interest are extracted into a solid phase.44 The use of SPE results in a much 'cleaner' sample, as most compounds that do not

exhibit certain shared properties with the analytes of interest have been removed, including those that interfere with chromatographic separation and analysis using MS. This step greatly increases the reproducibility of the sample analysis.45 As the sample analytes are absorbed into a solid phase and are subsequently eluted back into a liquid phase, the samples can be concentrated, which increases their detectability.

2.3.4 Sample separation

In targeted approaches, separating bio-sample analytes with chromatography based on their physiochemical properties prior to feeding the eluent into the MS instrument eases the metabolites subsequent identification and quantification.[46] Importantly, the suppression of ion signals due to overlaps of molecular weight is addressed through first separating compounds with the same or very similar masses with other physicochemical properties, e.g. polarity.[47] Targeted metabolomics sample separation using LC platforms are performed by running the appropriately prepared fluid bio-sample through liquid chromatograph (LC) at a given temperature and mobile phase gradient. As they pass through the column, the metabolites in the mobile phase interact with the stationary phase differently, causing their retention time within the column to differ.[48] In the case of polar, non-ionic metabolites, such as amino acids, two column types are currently in use are particularly popular, reverse phase-liquid chromatography (RP-LC) and hydrophilic interaction- liquid chromatography (HILIC).[33] Of the two, RP-LC had been considered the gold standard for most metabolomics-type analyses, however HILIC has recently become increasingly popular. The use of RPLC typically involves running the extracted metabolites within an organic mobile phase through a stationary phase along a gradient of decreasing solvent polarity, which retains the metabolites based on their hydrophobicity. As some metabolites are highly polar, often a derivatization step of the metabolites is included in order to allow for the metabolite to enhance their interaction with the column, with the added non-polar region also aiding in subsequent ionization with ESI as well as compound detection using CID, discussed below.[49] On the other hand, when using HILIC, the extracted metabolites are run through an aqueous mobile phase with high organic solvent content along a gradient of increasing solvent polarity and are retained based on their hydrophilicity.[48] Here, an aqueous layer coats the column, and metabolites interact with the aqueous layer via hydrophilic interactions. This allows for improved separation of highly hydrophilic and polar compounds, though at the expense of inferior separability of highly non-polar compounds.[50]

2.3.5 Sample injection and ionization using ESI

After having run through either the HILIC or RP-LC, the eluent is fed into a tandem mass-spectrometer (MS/MS) via an ionization source. Currently, clinical metabolite samples are most commonly ionized at atmospheric pressure using electrospray ionization (ESI).[31] Ionization using ESI is considered a 'soft ionization' technique, which helps prevent metabolite fragmentation caused by more traditional ionization techniques.[33] Essentially, the eluent is fed through a thin nozzle that produces a certain voltage, which causes the liquid in the nozzle to spontaneously form a cone shape, known as the Taylor cone [51]. The charge density of the liquid increases towards the tip of the cone, which competes with the surface tension force of the liquid. As the charge exceeds the Rayleigh stability limit, the surface tension of the liquid can no longer contain the charge and homogenous droplets are formed, which pass through the nozzle as a fine spray. [51] After the droplets pass through the ionization chamber, they are evaporated, often with the help of a nebulizing gas, such as helium. The analyte is subsequently ionized as it is released from the aqueous droplet while it evaporates during its passage through the ionization chamber into the MS inlet.[51] However, bio-samples are highly complex analytical matrices. They contain non-volatile contaminants, such as salts and other ionic or highly hydrophilic compounds, which when found within a droplet containing a compound that is more hydrophobic, it is more willing to receive the charge from the droplet.[50] As the more hydrophobic compound is not ionized, it hence is undetectable with the MS as such. This is addressed by combing ESI with a MS/MS system,[52] which has become a popular tool in clinical metabolism-related experiments.[18]

2.3.6 Sample analyte detection with triple quadrupole MS/MS

The triple quadrupole tandem mass spectrometer (QqQ) is essentially two mass filters connected to detectors, with a collision chamber between the two.[53] There are several alternatives for how to combine the three quadrupoles, known as scan modes, whose adequacy depends on the nature of the analysis. In targeted metabolomics analysis, as the metabolites of interest have been pre-defined, selected reaction monitoring (SRM), a form of multiple reaction monitoring (MRM) is the preferred scan mode, as it allows for the required sensitivity and selectivity for the analysis of small metabolites.[54] In general, the scan modes operate by allowing the ionized particles to enter the MS inlet, where they encounter the first quadrupole (Q_1) which scans through a range of radio frequency and direct current potentials and only allows particles within a certain range of mass-to-charge (m/z) to maintain a stable trajectory through the Q_1.[53] The Q_1 hence 'selects' precursor ions that can be

introduced to the collision chamber, which is the next quadrupole, q_2. Here the 'precursor' ions are forced to collide with inert gaseous atoms, which subsequently causes them to fragment. The resulting fragments, 'product ions', are then detected by the third quadrupole, Q_3. As the relative strength of bonds within the precursor ion are known, and fragmentation occurs at those bonds where the vibrational energy added exceeds the bond strength, it will fragment predictably, allowing for the fragments to help reliably identify the precursor ion.[55]

2.3.7 Raw spectral data processing

After the samples have been run through the MS/MS, the peaks of the raw spectral data must be analyzed in order to detect and quantify the targeted metabolites. Essentially, for scan modes such as SRM, metabolites of interest are identified by comparing the MS peak intensities from Q_1 and Q_3, as well as their chromatographic retention time.[21] The identified metabolites of interest are then quantifiable by comparing their spectra to their respective IS spectra. Spectral data processing can be broken down into feature alignment steps followed by peak picking steps.[33] Feature alignment is required as it has been well established that as a batch of samples are run through the chromatographic column, chromatographic retention time 'drift' occurs. The second step is to filter; here the common features are identified between samples, then compared with spectral databases. [56] The third step is to quantify the identified compounds through calibrating with the help of the IS, as well as check individual sample integrity with the help of pooled samples.

2.3.8 Processed metabolite data processing using statistical software

Once the raw MS spectral data has been processed, the resulting data should represent the absolute concentrations of the targeted metabolites in the sample. This processed data can hence be used to test experimental hypothesis by performing statistical analysis of the data through the use of statistical models best suited to answer the research hypothesis. Some considerations for choosing the appropriate statistical model include the nature of the variables tested, the sample size and number of cohorts, as well as how time factors into the experiment. Common approaches to interpreting processed targeted metabolomic data include the use of both univariate-based and multivariate-based statistical models.[57] Although the field is rapidly evolving, popular protocols and workflows exist and their use has been widely adopted.[21,58,59] Prior to their application to appropriate statistical models, the workflow generally deals with any missing values by either removing the metabolite altogether or replaced using

one of several possible methods for dealing with missing values.[52] Depending on the range of metabolite concentrations in the dataset, a normalization step is often included in order to better compare cohorts with widely ranging metabolite concentrations simultaneously.[59]

2.3.9 Targeted metabolomics is outsourced to ensure experimental reliability

As for any metabolomics experiment, the key to successfully using the UPLC-MS/MS based platform for targeted metabolomics experiments lies in the ability to create reproducible results. This is achieved by optimizing the sample preparation procedure, as well as by using standardized experimental procedures and materials.[21] As a result, to prevent errors in the experimental workflow, currently dedicated metabolomics 'centers' are used to outsource the laboratory portion of the work.[27,31]

2.4 Targeted metabolomics may continue to provide novel insight for clinical research

Following the advent of the genomics era that followed the human genome project around two decades ago, Hood[26] suggested applying metabolomics to elucidate details regarding the relationship between diet and health. It had already then been firmly established that myriad chronic pathologies are either the direct result of or correlate extensively to metabolic imbalances that are observable in biofluids and tissues.[19] When applied to a clinical setting, targeted metabolomics hence allows for the study of how precise biofluid metabolite concentrations relate to clinical phenotypes.[40] In mammals, metabolic processes are highly dynamic and can occur over the matter of seconds, or over many months, and criteria have been developed in order to take temporal and dynamic metabolic processes into account in clinical experiments.[60] However, it has been demonstrated that the majority of human serum metabolites are kept surprisingly stable,[61] a finding which greatly benefits research regarding how well, as well as to what extent metabolite profiles reflect the overall health status of the individual.[40] This finding is likely reflected in canines as well, given their similarities,[62] though to my best knowledge studies on stability of biofluid metabolites in canines has not been reported.

2.4.1 Metabolomics for studying the relationships between metabolism, health and diet in canines

Even small persistent metabolic imbalances due to environmental factors, such as diet, are thought to be a root cause of many of the chronic pathologies that humans[63] and their pet dogs suffer from.[64] Currently, several thousands of clinical metabolomics-based experiments have been reported.[29]

However, the most recent review that focuses on clinical metabolomics-based canine studies shows that, although its popularity is accelerating, the popularity of using metabolomics-based studies of canines still remains relatively low.[37] According to that review, 16 studies, i.e. approximately 43% of the metabolomics-based experiments utilized a quantitative approach, where 26% of studies performed targeted metabolomics and 17% performed metabolic 'profiling' of certain classes of compounds. Of the 16 studies reported, the popularity of using urine (7/16) serum (5/16) and plasma (5/16) samples for analyses were quite similar.[37] Of these, only two studies focused on the interaction between metabolism and diet, and five studies focused on the interaction between metabolism and a chronic pathology. Regarding diet, one study performed a metabolic analysis of blood serum using both GC-MS and LC-MS.[65] The other study performed a non-targeted analysis of urine and fecal samples using GC-MS and LC-MS, although the blood serum was also analyzed using a standard clinical biochemistry panel.[66] Of the five studies that regarded chronic pathologies, none studied or even controlled for the possible interactions between diet and subsequent metabolic modulation of the disease phenotype.[67-71] To my best knowledge, no study has used a targeted metabolomics approach to study the interactions between canine metabolism, diet, and an underlying chronic disease state. Furthermore, of the studies that focused on diet, no study focused on both the metabolome of urine and blood. However, the study of the effects of nutritional intake on a canine's blood serum biochemistry can be complemented with the simultaneous analysis of the metabolomic profile of the urine. An excess of a polar metabolite's concentration in blood above the needs of an organism's normal function can be seen as an increase in the metabolite concentration in the urine as it exceeds the renal threshold for that compound.[72] Although a few studies combined either blood serum or urine samples with other biosamples, e.g. serum with bile,[73] feces with urine,[74] only one study had analyzed both canine serum and urine samples simultaneously.[71] Although that study focused on intestinal dysbiosis, the authors did not study the effects of nor control for the possible effects of diet in their experiment. To our best knowledge, a metabolomics-based analysis of dog's serum and urine in response to diet has not been reported.[38,42]

2.5 Using dogs as model organisms has noteworthy advantages

Nutrition-based experiments have been notoriously difficult to perform on humans;[75] it is costly to include a sufficient amount of participants over sufficiently long periods of time. More importantly, controlling dietary intake of humans has shown to be challenging especially if the participants are not

housed throughout the study at a clinic, which is mainly due to that humans often lie about their food intake, or have difficulty quantifying everything consumed over any meaningful period of time.[76] As a result, much research ultimately aimed towards humans has been performed using 'traditional' model organisms, e.g. rodents and other small invertebrates.[77] There are noteworthy reasons why this is the case; they are cheap to breed and study, their lifespans are relatively short, genetic variance between individuals can be far more tightly regulated, and protocols for their use as model organisms have been well established.[77,78] However, within the realm of clinical nutritional metabolomics, using pet canines as a model organism instead of mice and rats has several noteworthy benefits and hence there has been a recent trend in clinical settings towards embracing canines as a model organism for humans.[64,79] Mice and rats are nocturnes and often studied in cages that do not reflect a typical human environment. Within that environment, mice poorly reflect how humans respond to environmental factors. For example, a recent review reported that only 5% of cancer drugs that showed promising results in mice were also shown to show efficacy and safety in humans.[80] Dogs on the other hand, are genetically,[62,81] epigenetically,[82] physiologically and behaviorally[83] closer to humans than mice and other rodents. Dogs share their environment with their owners and are typically regarded as family members.[37] Their lifestyle closely resembles that of their owners, including the same drinking water, in-house and outside exposures to toxins, and often even the same type of lifestyle. Epigenetically, they age similarly, but on a far shorter timescale than humans, allowing for the progression of shared chronic pathologies to be studied on a shorter timescale.[82] Their diets are easily controlled by their owners, and the owner is not incentivized to lie about their dog's food intake. The lack of a controlled environment that the laboratory cage offers is traded in for studying an organism in the very environment that both the dog and the human share. Conducting research in this less controlled environment undoubtedly increases the amount of background 'noise' in the data gathered, but so too is addressed through increasing the amount of data collected with the help of 'omics'-based approaches.[64,79]

2.6 Diet

As for any invertebrate, a dog's main exogenous source of metabolites is diet, and hence the diet's composition in large part influences the dog's blood metabolome, both directly and via the microbiota.[84] The majority of domesticated dogs in the developed world eat a kibble diet (KD).[85,86] According to the recently embraced NOVA food classification,[87-89] kibble is an 'ultra-processed' product. Kibble is a mixture of ultra-processed grains such as wheat, corn, and/or rice, mixed with

ultra-processed animal by-product meal and enriched with chemical additives, including synthetic vitamins, minerals, trace elements, preservatives, coloring agents, and palatability enhancers.[90,91] The raw meat-based diet (RMBD) in contrast, consists of raw animal parts. Complete and balanced commercial RMBDs also contain small amounts of raw vegetal matter as a source of fiber and sometimes a commercial premix of vitamins, minerals, and trace elements.[92,93] The popularity of RMBDs is particularly high in Finland,[94] but has also increased throughout the industrialized world.[95] The possible health benefits of feeding dogs with RMBDs remain understudied in comparison to its popularity.[92] In a recent review regarding the subject of raw feeding and its health effects,[85] the authors concluded that there was insufficient evidence to evaluate the risks and benefits of RMBDs with regard to canine health. The NOVA classification of RMBDs is currently under debate, and has hence not yet been established. Although the raw ingredients themselves are minimally processed[89] (chopped, mixed and frozen), minerals and vitamins are often added. The processing of the individual ingredients used to produce kibble may significantly alter their nutritional value and the overall health of the dog, although the reasons for this remains poorly understood.[96,97] The KD macronutrient profile differs remarkably from the RMBD profile. In terms of percent dry matter, a KD usually consists of a 'Protein: Fat: Carbohydrate' (PFC) macronutrient ratio 16-38:6-18:40-60%, whereas the PFC ratio of RMBD is typically 45:50:0-10%.[98]

2.7 Canine Atopic Dermatitis

Canine atopic dermatitis (CAD), part of the atopic complex, is a common systemic disease in canines, and is considered a form of chronic inflammation and manifests as an allergic response to an environmental factor which causes pruritus of the skin.[99] Clinical protocols for CAD diagnoses include the CADESI-4 scale and Favrot's criteria.[100,101] The development of CAD has been suggested to be genetically predisposed in canines, as well as further modulated by epigenetic factors.[99] Phenotypically the disease manifests itself differently in each individual, [102] although there is a relatively consistent trait of elevated concentrations of the antibody IgE across both atopy types and species.[103,104] Atopic dermatitis (AD) has been associated with several of the classic markers of metabolic syndrome (MetS) found both in humans [105] and in canines.[106] This relationship, likely mediated via inflammatory markers, is not fully understood.[107] The relationship between skin inflammation and oxidative stress markers in humans as a result of MetS has been studied,[108] and several pathophysiological disease mechanisms which combine AD and MetS have been proposed.[109,110] Nutrition has been shown to have

a vital role in determining the development of MetS through modulating metabolic pathways that have been attributed to the development of AD.[103] CAD typically comprises both food-induced atopic dermatitis and non-food-induced atopic dermatitis.[104] Although physiologically indistinguishable,[111] they can be differentiated with the diet-restriction provocation trial.[101] The link between metabolic health and CAD remains poorly studied. Most attention has focused on metabolic processes in the skin, especially in relation to fatty acids and lipids.[112-115] It has long been known that the immune system of animals can be modulated by metabolites derived from nutrition.[116] In canines for example vitamin D,[117] and fatty acid supplementations[118-120] have been shown to have a protective effect against allergic pruritic responses.

3 Experiment

3.1 Materials and methods

3.1.1 Animals and study design

A flowchart of the diet intervention is shown in Figure 2. In this diet intervention study, initiated in 2013, client-owned pet Staffordshire Bull Terriers were first studied with nutrigenomic [2] and hematological[3] approaches. The family history of the dogs has been reported elsewhere [2]. The diet intervention included inclusion, baseline, and end visits during the diet trial. No special inclusion diet was required prior to baseline, although the diet of each dog prior to their baseline visit was determined using a food frequency questionnaire.

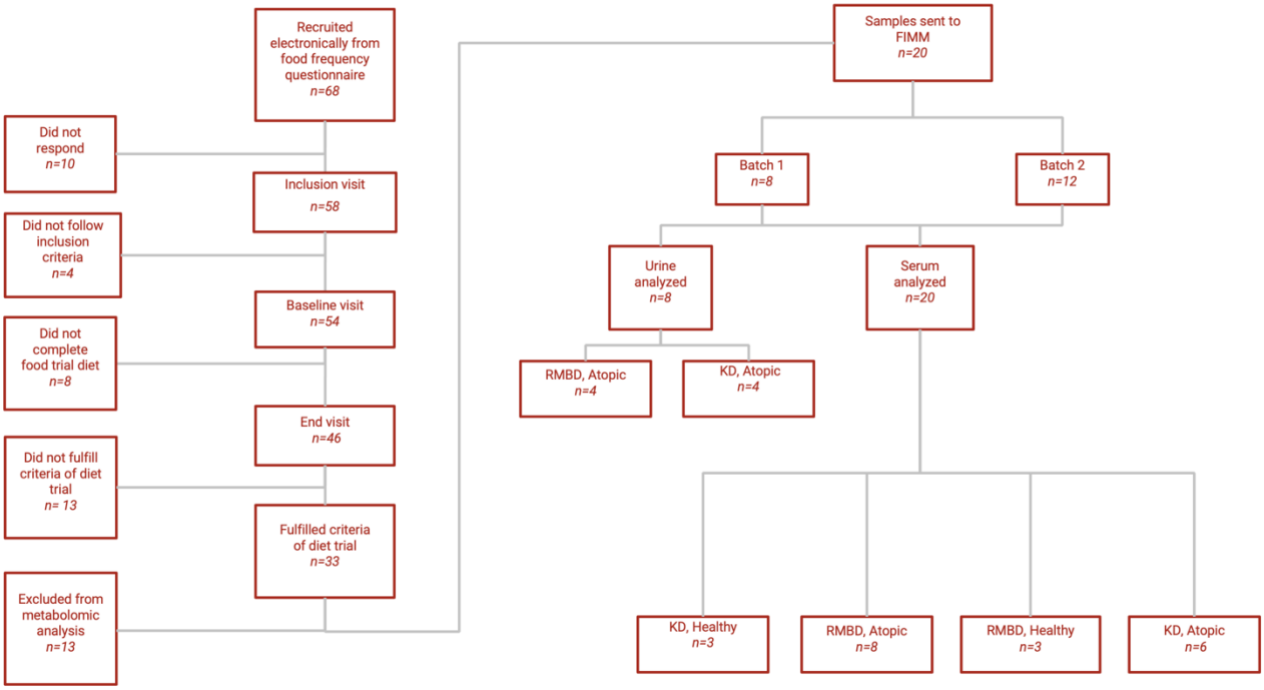

Figure 2. **Flowchart of study**: A flowchart depicting the selection process of the Staffordshire Bull Terriers used for the metabolomic analysis (n=20), and how they resulted in the cohorts based on diet (KD= kibble diet, RMBD= raw meat-based diet) and health status (CAD= canine atopic dermatitis).

Of the original cohort of Staffordshire Bull Terriers that underwent the whole study and fulfilled all criteria of the diet trial (n=46), only a subset (n=20) were selected for serum metabolomic analysis due to high running costs. The subset (n=20) was stratified based on owner-reported diets prior to baseline, as well as their diet during the study. All dogs analyzed for this study were fed solely kibble (KD) or raw food (RMBD) over a diet intervention period of 3-5 months (median =135 days) i.e. forming a KD cohort (n=9) and an RMBD cohort (n=11). The dogs included in the analysis (n=20) were also split into cohorts based on whether they were CAD-diagnosed (n=14), or healthy (n=6). For analysis that considered diet and health condition, the dogs were divided into four cohorts, Healthy-KD (n=3), CAD-KD (n=6), Healthy-RMBD (n=3), CAD-RMBD (n=8). Urine metabolomic analysis of samples collected at the end of the diet intervention was performed for a subset (n=8) of only CAD-diagnosed individuals, also due to high costs of analysis. The baseline samples were collected during September and October, and the end samples were all collected between February and April. The winter months were chosen for the diet intervention due to the seasonality of the disease, as CAD symptoms have been reported to be exacerbated as a result of pollen and blooming plant exposure [100,126]. Due to unrelated circumstances (pregnancy of the study co-ordinator) the trial ended later than planned.

Seasonality possibly affected the disease phenotype, as the end visit was delayed in some cases to spring, when plants already started blooming in Finland.

The canines were evaluated before and after the diet intervention by a dermatologist, who used Favrot's criteria [111], the Canine Atopic Dermatitis Extent and Severity Index (CADESI-4) scale [101], biochemical and hematological tests. The threshold for whether a canine suffered from CAD required a fulfilment of 5 out of 8 of Favrot's criteria. The severity of the CAD was diagnosed using the CADESI-4 scale, which categorizes CAD severity as follows: 0-10 = in remission, 11-33 = mild CAD, 34-59= moderate CAD, ≥60 = severe CAD. Owner-reported data regarding CAD severity as a visual analogue scale to evaluate the level of pruritus at two week intervals from baseline to end was also collected. The owner-reported pruritus conflicted with the dermatologist's CAD severity evaluation in some cases. However, for clarity, only the diagnosis reported by the dermatologist was used in this study.

The diets used in the study were a commercial kibble diet (KD), and two commercial raw meat-based diets (RMBDs). The RMBDs used in this study had an average PFC macronutrient ratio of 26:74:0 percent metabolizable energy (% ME). The KD diet used in this study had a PFC macronutrient ratio of 23:36:41% ME (Table 1).

Table 1. Percent metabolizable energy (%ME) of the kibble (Hill's Science Plan) and two raw-meat based diets (Mush BARF Vaisto, pork-chicken-lamb, beef-turkey-salmon). The values are calculated using the modified Atwater factors as suggested by the National Research Council [127].

Macronutrient	Hill's Science Plan™ Canine, adult sensitive skin with chicken (%ME)[1]	MUSH Vaisto (Pork-Chicken-Lamb) (%ME)	MUSH Vaisto (Beef-Turkey-Salmon) (%ME)	MUSH diets combined average (%ME)
Protein	23.28	23.84	28.09	25.96
Fat	35.76	76.16	71.91	74.04
Carbohydrate	40.95	0.00	0.00	0.00

[1] **%ME= % metabolizable energy**

The commercial dry diet used in this study was Hill's Science Plan™ Canine Adult Sensitive Skin with Chicken (detailed composition shown in Table S1a). The two commercial raw meat diets used in this study were MUSH Vaisto® Pork-Chicken-Lamb and MUSH Vaisto® Beef-Turkey-Salmon (detailed compositions shown in Table S1b). For the RMBDs, owners were free to choose either one or combine both diets. According to manufacturer claims, both the KD and the two RMBD options were 'complete diets'. Owners were asked to feed their dogs 99.9% with the trial food using amounts recommended by the manufacturer, adjusting amounts if their dog's bodyweight would start to fall or rise. Owners reported the adherence to diet using a food diary. Water was allowed ad libitum.

3.1.2 Samples

The metabolomic analysis of blood and urine samples were performed in two batches, i.e, *batch 1* and *batch 2*. Both batches are described in Table 2. For batch 1, blood serum samples, collected at baseline and end, and urine samples collected only at end, from atopic dogs (n=8) were used. For batch 2, only blood serum samples collected at baseline and end from a cohort of both atopic and healthy dogs were used (atopic n=6, healthy n=6). For analysis of serum, batches 1 and 2 were combined (atopic n=14, healthy n=6) for several of the analyses described below.

Table 2: Overview of the experimental setup of diet intervention, including division of Staffordshire Bull Terriers into diet cohorts (diet overview in Table 1), gender, health status, disease phenotype, diet intervention length, and age.

Batch	**1**		**2**		**1&2**	
Diet cohort	RMBD[1]	KD[2]	RMBD	KD	RMBD	KD
Dogs (total) (n)	4	4	7	5	11	9
Gender (male/female)	4/0	2/2	3/4	3/2	7/4	5/4
Sterilized (yes/no)	2/2	3/1	2/5	1/4	4/7	4/5
Blood serum analyzed	yes	yes	yes	yes	yes	yes
Urine analyzed	yes	yes	no	no	no	no
Atopy (total)(n)	4	4	4	2	8	6
NFIAD[3]/FIAD[4]	3/1	3/1	4/0	2/0	7/1	5/1
Healthy (n)	0	0	3	3	3	3
Mean diet intervention length (days) (SD[5])	126 (35.3)	141 (26.6)	137 (27.0)	136 (29.7)	133 (29.0)	139 (26.7)
Mean CADESI score at BL[6] (SD)	CAD: 13.5 (9.0)	CAD: 19.0 (10.8)	CAD: 12.5 (8.7) Healthy: 3.3 (1.2)	CAD: 18.5 (16.3) Healthy: 2.7 (1.2)	CAD: 13 (8.3) Healthy: 3.3 (1.2)	CAD: 18.8 (11.1) Healthy: 2.7 (1.2)
Age at BL (months; mean, SD)	44.7 (34.9)	56.2 (31.7)	60.8 (35.9)	75.2 (46.1)	54.9 (34.7)	66.8 (39.3)

[1]RMBD= raw meat-based diet; [2]KD= kibble diet; [3]NFIAD= non food-induced atopic dermatitis; [4]FIAD= food-induced atopic dermatitis; [5]SD= standard deviation; [6]BL=baseline

Blood samples were collected from the jugular vein using Vacuette® 3 mL EDTA, 3 mL lithium heparin, and 6 mL plain serum tubes by a closed method (Vacutainer® Safety-Lok™ Blood collection sets, Becton Dickinson, Meylan, France). Serum samples were allowed to clot at room temperature for 30 minutes before centrifugation (2100 x g for 15 min). Urine samples were collected into factory-clean specimen jars and frozen after collection in 5 mL tubes. All samples were fasting samples collected in the morning. After collection they were stored at -80 °C.

The targeted metabolomic analysis of the dogs' serum samples at baseline and end of the diet intervention (all dogs n=20, healthy n=6, atopic n=14) were performed at the Finnish Institute of Molecular Medicine (FIMM) using targeted liquid-chromatography mass spectrometry. As targeted

metabolomics of canine samples had not been performed before the first batch (Batch 1, *n*=8) was sent to FIMM to test the method. As the results were interpretable, more samples (Batch 2, *n*=12) were sent. Common polar, non-ionic metabolites (*n*=102) were targeted with nanomolar accuracy (±0.005µM) using the BioCrates p180 kit as standards for isotopic quantification. A full list of the targeted metabolites used in the standard mixture are included in the supplementary material (Supplementary file 20). A labeled internal standard mixture (10 µL) was added to 100 µL of serum or urine samples, which were all run in triplicate to ensure reliability. Metabolites were extracted by adding 4 parts (1:4, sample: extraction solvent) of the 100% acetonitrile + 1% formic acid solvent. The collected extracts were dispensed into Ostro^{TM} 96-well plates (Waters Corporation, Milford, USA) and filtered by applying vacuum at a delta pressure of 300-400 mbar for 2.5 min using a robotic vacuum station. The filtrate was transferred to a 96-well collection plate, which was placed under the Ostro^{TM} plate. The collection plate was sealed with the well cap mat and placed in the auto-sampler of the liquid chromatography system for injection. Samples were analyzed using high-throughput targeted quantitative metabolic profiling using the ACQUITY UPLC-MS/MS instrument (Waters), with a 1.7 µm BEH amide HILIC column for chromatography.

3.2 Data pre-processing

Sample preparation for UPLC-MS/MS, as well as raw spectral data processing, was carried out on site by FIMM personnel. Subsequent concentration data were provided for each metabolite, along with comments regarding their reliability. The raw spectral data was acquired with MassLynx 4.1, and TargetLynx software. Detailed information regarding the raw spectrum metabolomics analysis can be found elsewhere.[128] All metabolomics instrumentation used for analysis was owned by and located in the FIMM metabolomics unit in Biomedicum (Metabolomics Unit, Finnish Institute for Molecular Medicine FIMM, Helsinki-00014, Finland).

Based on LC-MS raw data processing, for batch 1, 80 of the original 102 targeted metabolites in serum samples (Table S2a), and 80 of the original 102 metabolites in urine samples, were used in the statistical analysis (Table S2b). The raw data from batch 2 were considerably better, and only one of the 102 metabolites, spermidine, had to be omitted from analysis. For the combined batch serum analysis, 79 of the original 102 metabolites were used for the statistical analysis (Table S2c).

Original metabolite values in the serum and urine datasets were reported in µmol/L. Urine metabolite values were normalized to their respective creatinine concentrations. Urine metabolite values used in data analysis were adjusted to metabolite(µmol)/creatinine(mmol). Creatinine-adjusted urine

metabolite values were used in the analysis that combines serum and urine datasets. Only usable metabolite concentration values found in both datasets were used. In summary 72 of the original 102 metabolite values were used in the analysis that combines serum and urine metabolite values.

3.3 Statistical Analysis

Statistical analysis was performed with the R package MetaboAnalystR.[58] Source code for the statistical analysis workflow was documented as R-generated analysis reports (Supplementary files 1-19). Targeted metabolites that were unreliably quantified or contained over 50% missing values were removed with Excel prior to data processing with R. The integrity of all serum samples and urine samples were checked with R prior to data analysis. As metabolites concentrations fluctuate greatly, the raw concentration values in both serum and urine were log transformed using a generalized logarithm function, allowing the concentrations to assume a more normal distribution for subsequent analysis. To improve the sample size and hence statistical power for downstream analysis, batch correction for the end-of-diet time points of batch 1 and batch 2 serum data was performed using the ComBat empirical Bayes method developed by Johnson et al. (2007)[129] in order to combine the two cohorts as there was significant variation due to batch effect. Combined-batch analysis of serum concentrations from batches 1 and 2 used values generated with the K-nearest neighbor algorithm prior to their combination to estimate any remaining missing values. The similarity between batches 1 and 2 end values was analyzed with principal component analysis. A 2-D principal component analysis plot of both pre- and post-correction is attached in the appendix (Figure S1). Each metabolite included in the combined batch analysis was tested to see whether there was a significant difference between batches after batch correction using a t-test. No significant differences were observed due to batch after the batch correction was performed. In all of the metabolite datasets used in this study, the K-nearest neighbor algorithm was used to compute missing metabolite values for metabolites that were missing less than 50% of the values within each cohort

For the results of statistical analysis, the cutoff for significance was set at FDR<0.05 (False Discovery Rate, also referred to as the FDR-adjusted p-value or q-value in some tables). In all statistical analyses, p-values are reported. As a general rule for metabolomics analysis, the reporting of FDR-values are recommended to ensure that results are statistically significant as the number of parameters tested are far greater than the number of samples.[59] In essence, the FDR 'controls the expected proportion of falsely rejected hypotheses'.[130]

3.3.1 Univariate analysis of baseline and end of diet intervention

Univariate analysis of baseline serum values from batch 1 and batch 2, as well as the combined batch dataset with respect to diet cohorts and health status cohorts, was performed to confirm whether there were any significant metabolite concentration differences between either cohort at the baseline of the diet intervention. Analyses of diet and health were first performed separately. For both the baseline and end of diet intervention, a general linear model (GLM), and parametric t-tests were used to observe statistically significant fold changes between the RMBD and KD cohorts in Batch 1 serum and urine samples, in Batch 2 serum samples, and in the combined batch serum samples i.e., analysis of all dogs in the study. Univariate analysis reports were created for each test between diet cohorts and health status both at the baseline and end of the diet intervention, and are can be found in the supplementary material (Supplementary file 21).

3.3.2 Univariate analysis of CADESI-4 score, weight, and age with diet

The change in CADESI-4 scores between diet cohorts was determined by testing the change (end timepoint minus baseline) to see whether diet correlated with change in phenotype. The same was done for weight and age. Changes in CADESI-4 scores were also compared within dietary cohorts between gender, as well as neutering status.

3.3.3 Analysis between sample media and dietary cohorts at end of diet intervention

A two-way analysis of variance (ANOVA) was performed between sample media (blood or urine) and dietary cohorts (KD or RMBD). Hierarchical clustering was then combined with the results from the two-way ANOVA to generate heatmap visualizations of the significantly different metabolites between diet cohorts and sample type in the serum and urine data. The differences in variance between cohorts are also reported as F-values.

Fold-change comparisons combined with t-tests were used to identify significant differences between serum and urine metabolite concentrations. The GLM was then used to perform correlation analysis between samples and identify which significant metabolites correlate with diet. To visualize how the samples within cohorts contributed to significant metabolite differences observed with the GLM, heatmap visualizations of significant metabolites (FDR<0.05) within individual batches, as well as combined batch results from Fisher's least significant difference (LSD) test were created.

To further explore the results seen from t-tests and the ANOVA, a supervised multivariate regression-based analysis, partial least squares-discriminant analysis (PLS-DA), was used to test the significance between sample media and diet cohorts. This was performed to determine the extent to which the linear combination of the metabolite values for a given sample can predict the diet cohort of the dog. For each component, each metabolite was assigned a variable importance in projection (VIP) score. The VIP score signifies the relative contribution a given metabolite has to discriminating the cohorts that are compared in the model and is dependent on the percentage variation explained by the component vectors used in the model.

To observe the risk of overfitting when using PLS-DA, cross-validation using the leave-one-out approach (LOOCV) was used to determine the accuracy, R2 and Q2 values of each respective component, where Q2 values have been computed to resemble the scale used for R2 and accuracy scores ($0< x <1$). Loading plots for the components 1 and 2 (the two components which explain the most variation between cohorts) were visualized to show the relative contributions metabolites had to the creation of their respective component vector.

3.3.4 Analysis between diet and atopy at end of diet intervention

Analysis of diet and health combined for batch 2 and combined batch datasets to test for interaction was also performed with a two-way ANOVA. As all dogs in batch 1 were diagnosed with atopy, no analysis with regards to health status was performed. For the combined batch dataset, the results from the end of the diet intervention were studied with a two-way ANOVA between diet and atopy and their interactions. Results were visualized with a heatmap. To further explore the results seen from t-tests and the ANOVA, PLS-DA was used to identify the extent to which the diet and atopy cohorts differed.

4 Results

4.1 Univariate analysis of baseline and end of diet intervention

By controlling for baseline bias, mildly significant concentration differences of arginine, histidine, and threonine between the two diet cohorts (p-value <0.05, FDR>0.05) were found (Table S4). No significant metabolite concentration differences between atopic and healthy individuals were observed either at baseline or at the end of the diet intervention.

For all dogs' serum samples in the study (n=20), the metabolites that significantly differ (FDR ≤0.05) between diet cohorts at the end of the diet intervention are presented in Table 3. A more comprehensive

table of all dogs at the end of the diet intervention, significant differences between the diet cohorts of the batches separately, only urine metabolites from the individuals of Batch 1 (n=8), as well as serum metabolites from only atopic dogs (n=14) are included in the appendix (Tables S5-S9).

Table 3. Comparison of significantly different metabolite concentrations in all dog's serum samples between kibble diet (KD, n=9) and raw meat-based diet (RMBD, n=11) at end of diet intervention. Mean serum concentrations are presented as the natural log of the original metabolite concentration.

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold change	In KD cohort
Methionine	6.686 (0.294)	5.697 (0.305)	< 0.0001	0	1.17	Up
4-Pyridoxic Acid	-8.830 (0.460)	-11.025 (0.804)	< 0.0001	0	-1.25	Up
Citrulline	5.659 (0.204)	4.654 (0.507)	< 0.0001	0.0011	1.22	Up
Cytosine	-4.146 (0.790)	-5.964 (0.930)	0.0002	0.0026	-1.44	Up
Proline	7.965 (0.406)	7.099 (0.403)	0.0002	0.0026	1.12	Up
Cystathionine	3.154 (1.292)	0.152 (1.004)	0.0002	0.0026	20.78	Up
Taurochenodeoxycholic Acid	-0.898 (0.762)	-3.255 (1.357)	0.0002	0.0026	-3.62	Up
Hexanoylcarnitine	-7.033 (0.484)	-5.937 (0.760)	0.0015	0.0148	1.18	Down
Decanoylcarnitine	-6.414 (0.485)	-5.443 (0.661)	0.0018	0.0156	1.18	Down
Glycine	8.629 (0.299)	8.049 (0.407)	0.0023	0.018	1.07	Up
Creatine	4.155 (0.616)	5.176 (0.753)	0.0043	0.0297	-1.25	Down
Kynurenine	0.849 (0.513)	0.242 (0.319)	0.0045	0.0297	3.51	Up
Dimethylglycine	2.369 (0.511)	1.606 (0.575)	0.0062	0.0374	1.48	Up
Trimethylamine-N-Oxide	-3.100 (11.157)	1.534 (0.830)	0.0074	0.042	0.49	Down

[1]SD= standard deviation; [2]KD= kibble diet; [3]RMBD= raw meat-based diet; [4]FDR<0.05= false discovery rate < 0.05

At the end of the diet intervention, hexanoylcarnitine (FDR=0.015, p=0.0015), decanoylcarnitine (FDR=0.016, p=0.0018), octanoylcarnitine (FDR=0.052, p=0.01), acetylcarnitine (FDR=0.086, p=0.021), creatine (FDR=0.03, p=0.005) and creatinine (FDR=0.15, p=0.041) concentrations were higher in serum of the RMBD cohort than in the KD cohort (all dogs, n=20). Higher serum concentrations of urea-cycle metabolites citrulline (FDR=0.001, p<0.0001) and proline (FDR=0.002, p=0.0002), the nucleobase cytosine (FDR=0.0026, p=0.0002) were observed in all of the dogs of the KD cohort. Higher concentrations of the primary bile acid taurochenodeoxycholic acid (FDR=0.0026,

p=0.0002), and taurocholic acid were found in the KD cohort relative to the RMBD cohort (1.87-fold higher concentration, FDR=0.112, p=0.028). Serum methionine concentrations were higher in the KD-fed dogs (FDR<0.0001, p<0.0001), as well as cystathionine (FDR=0.0026, p=0.0002), dimethylglycine (FDR=0.037, p=0.0062), and 4-pyridoxic acid (FDR<0.0001, p<0.0001). There were higher urine concentrations of betaine, the precursor to dimethylglycine, in the RMBD-fed cohort (FDR=0.0022, p=0.0008), as well as a trend in serum of all dogs (FDR=0.086, p=0.02). Notably, dogs from batch 1 in the KD cohort also had significantly higher urine concentrations of methionine (FDR<0.02, p<0.0002) and 4-pyridoxic acid (FDR<0.04, p<0.002) (Table S6). There were no metabolites that significantly differed between diet cohorts of the healthy individuals (KD n=3, RMBD n=3), although several metabolite concentrations differed with a p-value<0.05 (FDR>0.05, p<0.05) (Table S10).

4.2 Two-way ANOVA between sample media and diet at end of the diet intervention

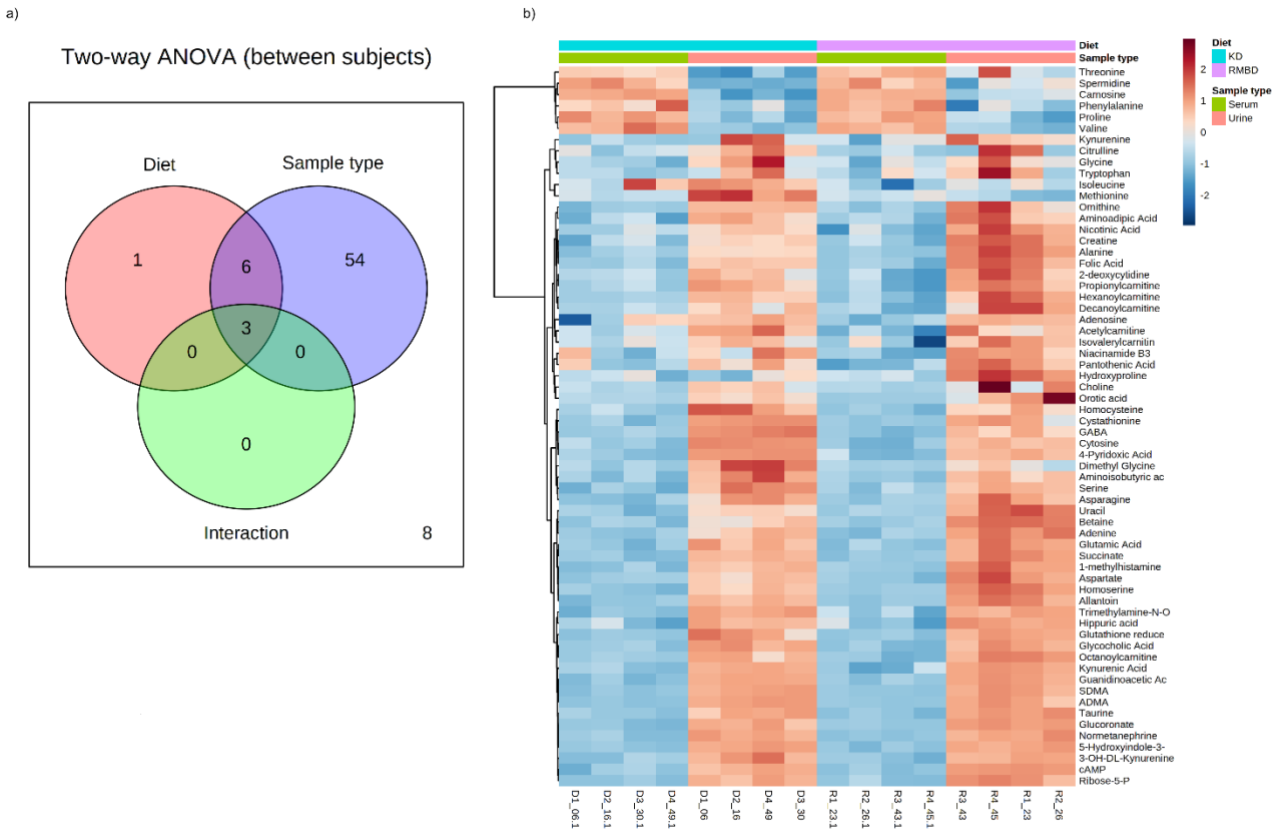

Figure 3. **Batch 1 (*n=8*) comparison of serum and urine profiles between diet cohorts** a) An overview of sample media and diet interaction at the end of diet intervention where metabolite values differ significantly (FDR<0.05) between diet cohorts (red) and sample type (blue), as well as interaction between the two (dark green and purple). b) A heatmap illustrating significant features from the two-way ANOVA. Values relative to the combined cohort average are represented as a color spectrum and have been scaled to -2 (blue) through 2 (red) (KD= kibble diet, RMBD= raw meat-based diet).

A two-way ANOVA was used to see whether any significant difference in serum metabolite concentrations between the diet cohorts could be seen in urine metabolite concentrations (Figure 3a). Out of the 63 metabolites that differed significantly between serum and urine, ten also differed between diet cohorts with interaction detected in five of the metabolites (Table S10). The significantly different metabolites between diet cohorts and sample type (serum and urine) from the two-way ANOVA were visualized with a heatmap (Figure 3b). To further explore how urine and serum samples differed between the diet cohorts of batch 1, a PLS-DA was performed. The parameters of the model, calculated with the LOOCV approach, are shown in Table S14a. Components 1 and 2 were plotted against each other (Figure 4) with shaded circles representing the 95% confidence interval area for the respective diet cohorts.

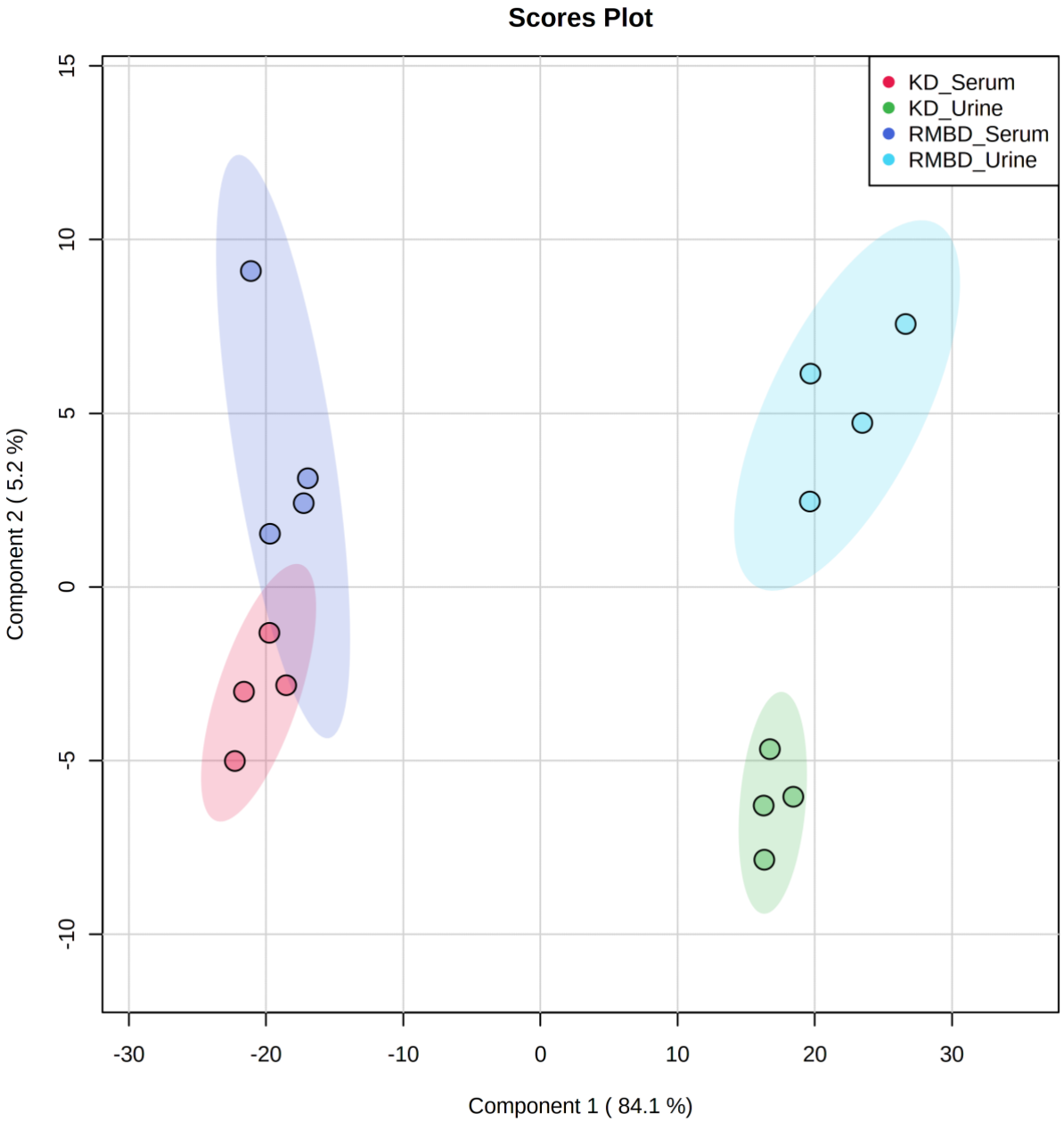

Figure 4. **PLS-DA shows how the serum and urine profiles of Batch 1 (n=8) can separate diet cohorts** PLS-DA of Batch 1 dogs (n=8) at the end of the diet intervention. Plot shows how serum, urine, and the KD (n=4) and RMBD (n=4) cohort metabolites differed at the end of diet intervention, shown with 95% confidence intervals (shaded regions) (KD= kibble diet, RMBD= eaw meat-based diet).

In the 2-D PLS-DA plot presented in Figure 4, the extent to how much within-cohort variation exists for diet cohorts and urine and serum samples was visualized. When the first two components of the PLS-DA were plotted against each other, the urine and serum samples were separable with the first component, and the RMBD and KD diet-cohorts were separable with the second component. However, likely due to the low sample size, the predictability of the model calculated with R2 and it predictability

when testing the model (Q2) was 0.108, and as such can be considered quite weak. However, although the Q2 is small, the model describes the extent to which the sample media accounts for most of the variance. There was a minor overlap of confidence intervals between diet cohorts observed in serum samples when separated with component 2.

4.3 Univariate analysis of CADESI-4 score, weight, and age with diet

According to the evaluation of CAD severity at the end of the diet intervention, neither the KD or the RMBD significantly changed the CADESI-4 score outcome of the CAD-diagnosed dogs. According to the diagnoses performed by the dermatologist, all CAD-diagnosed canines in this study suffered from mild CAD, and CAD severity remained mild in all individuals throughout the diet intervention period. The difference between diet cohorts was insignificant, with a weak worsening trend in the KD cohort ($p = 0.104$). There was a general trend in worsening of CADESI-4 scores found in both diet cohorts (for the KD n=9, $\mu=18.3$, $\sigma=13.8$), (for the RMBD n=11, $\mu=6.9$, $\sigma=6.5$). The change in CADESI-4 scores did not result in a progression from mild to moderate CAD symptoms in any of the CAD-diagnosed canines however. In the serum samples of dogs from all dogs (n=20), no significant weight and age differences between the KD and RMBD cohorts at the end of the diet intervention were detected. Results from the univariate analysis of CADESI-4, weight, and age across diet and disease cohorts are presented in Tables S3a-e.

4.4 Analysis between of diet and atopy

In all the atopic dogs, no significant differences in CADESI-4 scores between diet cohorts were found at the diet intervention baseline, where the dogs' diets were mixed, or at the end of the diet intervention. The outcome of serum concentrations of all dogs (n=20) at the end of the diet intervention were visualized as a two-way ANOVA between diet and atopy and their interactions (Figure 5a). Here, the RMBD and KD cohorts were classified as either healthy (Healthy-RMBD, n=3, Healthy-KD, n=3) or atopic (RMBD, n=8, KD, n=6). Metabolite values that differed significantly between either diet or health status cohorts, or their interaction, are presented in Table S12. The significantly different metabolites between diet cohorts from the two-way ANOVA of the atopic and healthy canines were visualized with a heatmap (Figure 5b).

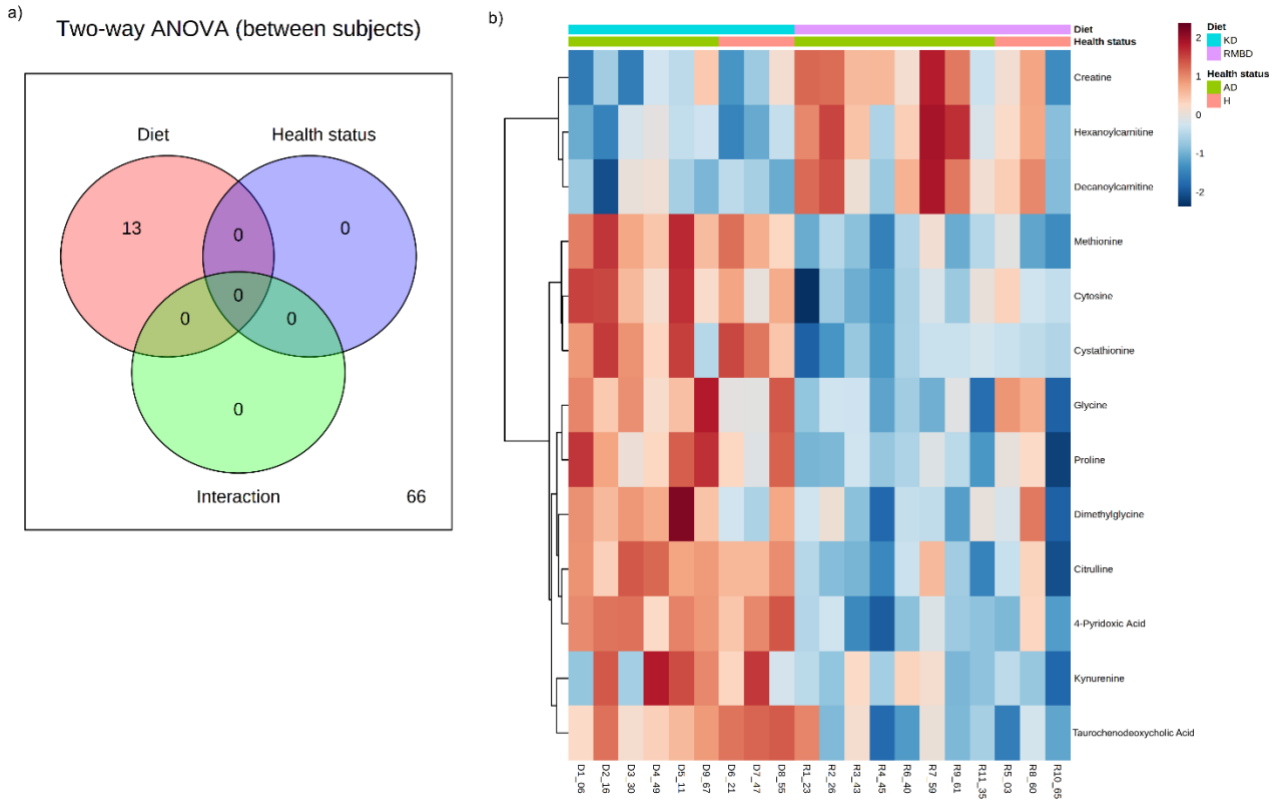

Figure 5. **ANOVA analysis of serum from all dogs at the end of diet intervention shows significant metabolite differences between diet cohorts, but not between health status cohorts.** a) An overview of how metabolite values differ significantly (FDR<0.05) between diet cohorts (red), and health status cohorts (blue), as well as any significant interaction between them (green) for all dogs (n=20) at end of diet intervention. b) A heatmap illustrating significant metabolite concentration differences in the two-way ANOVA for CAD-diagnosed (n=14) and healthy individuals (n=6) (green and orange), and between the kibble diet (KD) (n=9) and raw meat-based diet (RMBD) (n=11) cohorts at the end of diet intervention.

To further address the separation of cohorts based on diet and health status, PLS-DA analysis was performed to see how the metabolite profiles differed between diet and health status cohorts (Figure 6a). The parameters of the model were calculated using the LOOCV approach and are shown in Table S14b. Likely due to the low sample size, as well as the similarity between the CAD-diagnosed and healthy individuals serum metabolite concentrations, the predictability of the model calculated with R2 and it predictability when testing the model (Q2) was 0.277, which is relatively weak. Nevertheless, the model gives an indication towards how the healthy individuals in both diet groups were more closely clustered among themselves than the atopic individuals of either diet cohort. The top 20 VIP scores were visualized as a heatmap that looks at the top 20 metabolites across all components (Figure 6b), with which the diet cohorts could be separated, but that the health status cohorts (CAD-diagnosed and healthy) could not. Many of the metabolites found to be significantly different with the two-way

ANOVA described above, and the univariate analysis at end of the diet intervention (Table 3) were also found to have high VIP scores.

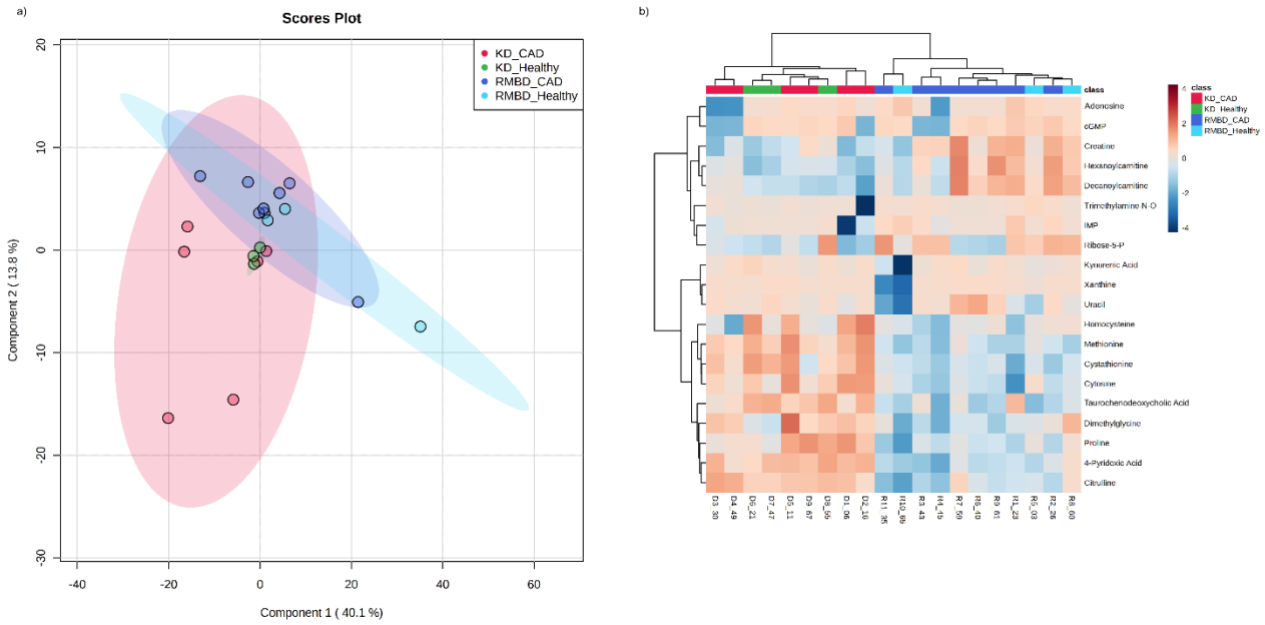

Figure 6. **PLS-DA analysis of the diet cohorts and health status cohorts.** A) PLS-DA (partial least squares-discriminant analysis) plot of the first two components, displayed with 95% confidence intervals for each diet group (shaded regions of same color). B) A PLS-DA VIP score heatmap visualization of the most important features (n=20) across components. (KD = kibble diet, RMBD = raw meat-based diet, CAD= canine atopic dermatitis).

As a follow up to the two-way ANOVA, an unprotected Fisher's LSD test was used to compare how the metabolite concentrations at the end of the diet intervention differed between the four cohorts, i.e. the healthy and atopic dogs of both diet cohorts. The significant differences (FDR<0.05) between these cohorts are presented in Figure 7 as group averages. The tabulated results are included in the appendix (Table S13).

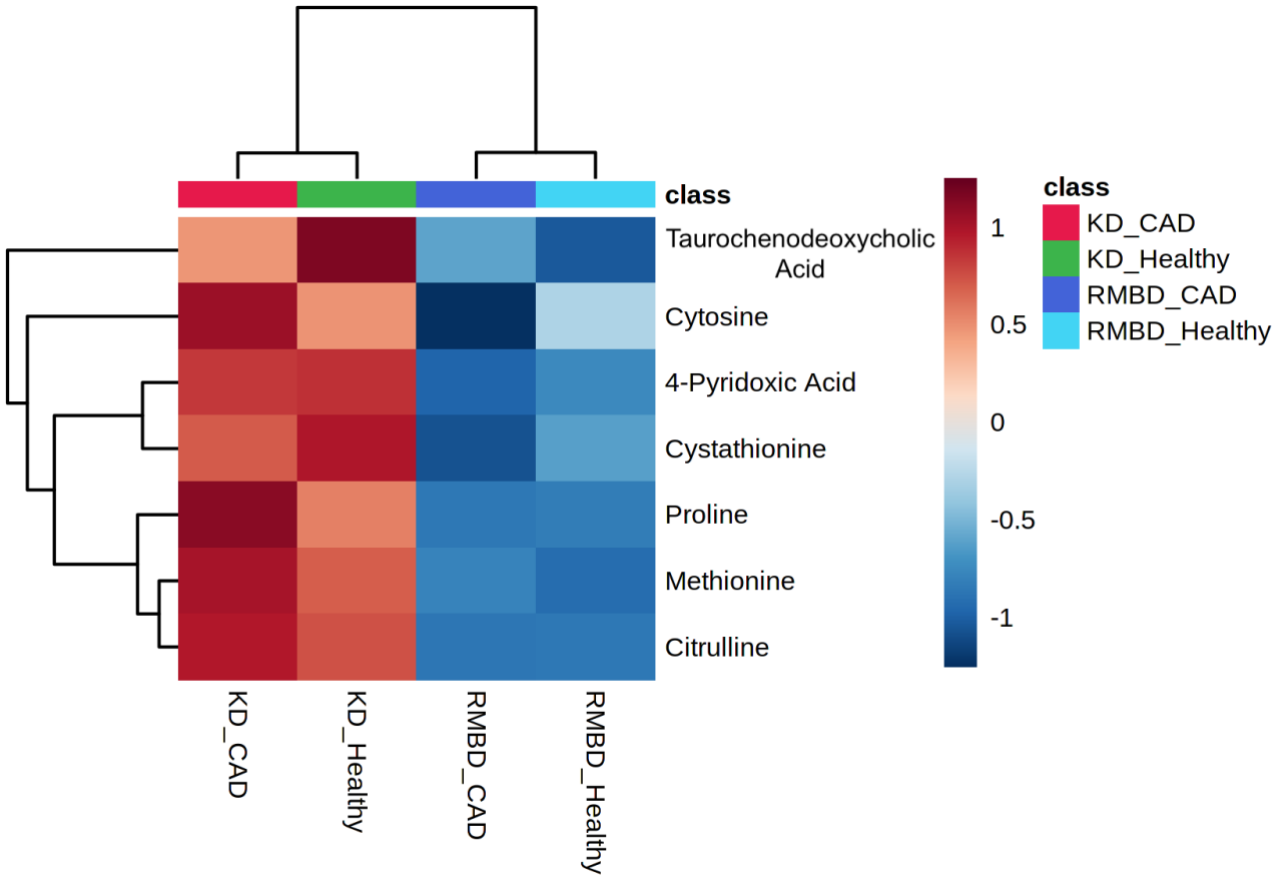

Figure 7. **Fisher's least significant difference (LSD) test to determine the significant differences between diet cohorts of healthy and atopic individuals.** Significant differences between metabolite concentrations calculated with Fisher's LSD test (KD= kibble diet, RMBD= raw meat-based diet, CAD= canine atopic dermatitis).

5 Discussion

5.1 Diet cohorts readily distinguished by distinct serum and urine metabolite profiles

The two diets included in this study were remarkably different in terms of the types of raw ingredients used, their macro- and micronutrient composition, and their manufacturing methods. This suggests that the feeding of a particular diet could have a profound impact on metabolism, which, in turn could have an effect on the dog's overall health and wellbeing. To our best knowledge, no data are available about the comparative study of blood and urine metabolomics in response to raw meat-based and kibble diets. Most of the metabolomics-based studies performed before 2015 are referred to in a review paper by Allaway (2015) [64]. To date, only the study by Schmidt et al. (2018) (7) compares the differences between a RMBD and a KD using metabolic profiling [131]. However, that study considers the fecal metabolome. The first study to evaluate health outcomes as a result of feeding commercial RMBDs

was published in 2012 [132]. The authors concluded that no undesirable changes occurred to either blood biogenic amine concentrations or skin and coat conditions in dogs fed the RMBDs in their study. Here, the major differences in metabolite concentrations observed between the diet cohorts could indicate impact on blood biochemistry, overall health, as well as the CAD condition are discussed in light of literature found regarding these topics.

There were higher concentrations of several of the carnitines, creatine and creatinine in the serum of the RMBD cohort than in the KD cohort (all dogs, n=20) (Table 3). This finding is likely reflected in the markedly higher meat content of the RMBD diet. Meat is the main dietary source of carnitines [133] and creatine [134]. It is likely that the elevated creatinine concentrations in the RMBD cohort because creatine is the direct precursor of creatinine [135]. Furthermore, carnitines play crucial roles in long-chain fatty acid transport for mitochondrial oxidation, which is to be expected of canines eating a fat-rich diet. Higher serum carnitine concentrations have been associated with anti-aging effects in canines [136]. The authors note that higher carnitine concentrations are associated with younger dogs, but they make no claims as to age-related health benefits [136].

The urea-cycle metabolites citrulline and proline were found in significantly lower serum concentrations in the RMBD cohort than in the KD cohort (Table 3). These metabolites are involved in urea production and ammonia recycling [137,138]. Citrulline is the direct precursor for arginine synthesis [139]. Meat protein contains high amounts of both arginine [140] and creatine [134], where arginine, and subsequently citrulline is required for creatine synthesis [141]. As citrulline is used to accept the amino groups of excess amino acids from dietary protein [142], the higher protein content in the RMBD may explain this observation, i.e. less citrulline would be required by the KD-fed dogs, which possibly explains the higher concentrations observed in the KD cohort. Proline is found in especially high concentrations in collagen [143], an unexpected finding considering the likely higher collagen content in the RMBD.

The significantly higher serum concentrations of the nucleobase cytosine observed in all of the dogs of the KD cohort (Table 3), as well as urine concentrations in the Batch 1 KD cohort (Table S6) at the end of the diet intervention, is notable. To the best of our knowledge no studies have investigated the relationships of diet between cytosine concentrations in blood and urine.

In blood serum of all dogs in the KD cohort, higher concentrations of the primary bile acid taurochenodeoxycholic acid could be seen after the diet intervention than in the RMBD cohort. Elevated concentrations of the downstream product of taurochenodeoxycholic acid, deoxycholic acid,

has been implicated in colon tumorigenesis in both mice and humans [144,145]. Colon cancer is exceptionally high in canines [146], although the links to bile acid concentrations remain poorly understood. Although insignificant, taurocholic acid was also found in higher serum concentrations in the KD cohort relative to the RMBD cohort. It has been established that the composition of the microbiota throughout the canine gut is largely defined by the nutritional profile of dietary intake [147,148]. The microbiota composition modulates the amount and composition of nutrients that are able to pass through the gut endothelium, hence affecting blood serum biochemistry [149]. Most studies on this topic have focused solely on fecal samples [150]. Bile acid concentrations have been suggested to be sensitive to changes in gut microbiota composition. It has been reported that fecal bile acid concentrations increase in canines when fed an animal-based, high-fat, low-fiber diet [151]. Elevated primary bile acid concentrations in blood have been shown to be a sign of elevated inflammation [152], especially with regards to the liver [153]. No reference values regarding what levels lead to increased inflammation has been reported for canines [151].

Due to their toxicity, bile acid concentrations are tightly regulated in mice [154], and furthermore are usually increased as a response to increased fat digestion [155] as they function essentially as emulsifiers to improve fat absorption through the endothelium. Given the far greater amounts of fat present in the RMBD this finding comes as a surprise. However, it should be noted that there were also large amounts of carbohydrate present in the high-fat, low-fiber diet in the study performed by O'Keefe et al. (2015) [155]. As the RMBD has little to no carbohydrate, the energy metabolism of the canines was likely markedly different from the humans participating in the diet interventions of the O'Keefe et al. (2015) [155] study. The RMBD-fed dogs were possibly even ketogenic, i.e. causing a switch over to increased β-oxidation of fatty acids as a primary means for ATP production [156]. It has been shown that even in the presence of high fat content, glucose is the preferred energy substrate in mammals [157]. Canines fed a high-fat diet, in particular one rich in medium-chain triglycerides (MCTs), even in the presence of high carbohydrate, have been reported to be ketogenic [158,159]. However, results from both studies performed by Law et al. (2016, 2018) [158,159] are questionable, as the authors neglected to measure or report ketone body values in the dogs and thereby establish whether the diets were ketogenic [160]. Furthermore, ketone body production has been shown to rely on low levels of carbohydrate [161]. Although MCTs are readily used for energy, even in the presence of carbohydrate [160], it does not necessarily switch the dog to a state of endogenous ketosis, i.e. where fat is the preferred metabolic energy source– the underlying assumption of a ketogenic diet [161]. Ketogenic diets may affect serum bile acid concentrations in mice [162]. In mammals, a switch over to ketogenic metabolism has major

implications for altering glycolytic energy metabolism [163], and an increase in NADPH production, which is produced via the pentose phosphate pathway [164]. In the RMBD cohort of batch 1 (n=4) (Table S7), a significantly higher level of ribose-5-phosphate was observed, indicating an upregulation of the pentose phosphate pathway [163], and subsequently a downregulation of glycolysis and upregulation of ketone body production. However, higher concentrations ribose-5-phosphate were not observed in the RMBD cohort of batch 2 (n=7) or when observing all the dogs in the RMBD cohort (n=11). The discrepancy between batches in itself merits further investigation. The finding in batch 1 may indicate that the RMBD was ketogenic, although to date no studies to our knowledge have considered the ketogenic properties of RMBDs, an area that merits further investigation.

At the end of the diet intervention, all canines in the KD cohort had higher serum concentrations of the sulfur-containing amino acid methionine than the RMBD cohort (Table 3). The batch 1 KD cohort also had significantly higher urine methionine concentrations than the RMBD cohort (Table S6). The serum of all canines in the KD cohort had higher levels of cystathionine. Both play important roles in homocysteine metabolism via the remethylation pathway, via the transsulfuration pathway, and via one-carbon pathway.[165] The amino acid homocysteine is remethylated to methionine in a process dependent on vitamin B12 (B12) or is converted to cysteine via cystathionine in a vitamin B6-dependent process.[165] A schematic representation of the methionine and transsulfuration pathways are represented in Figure 8.

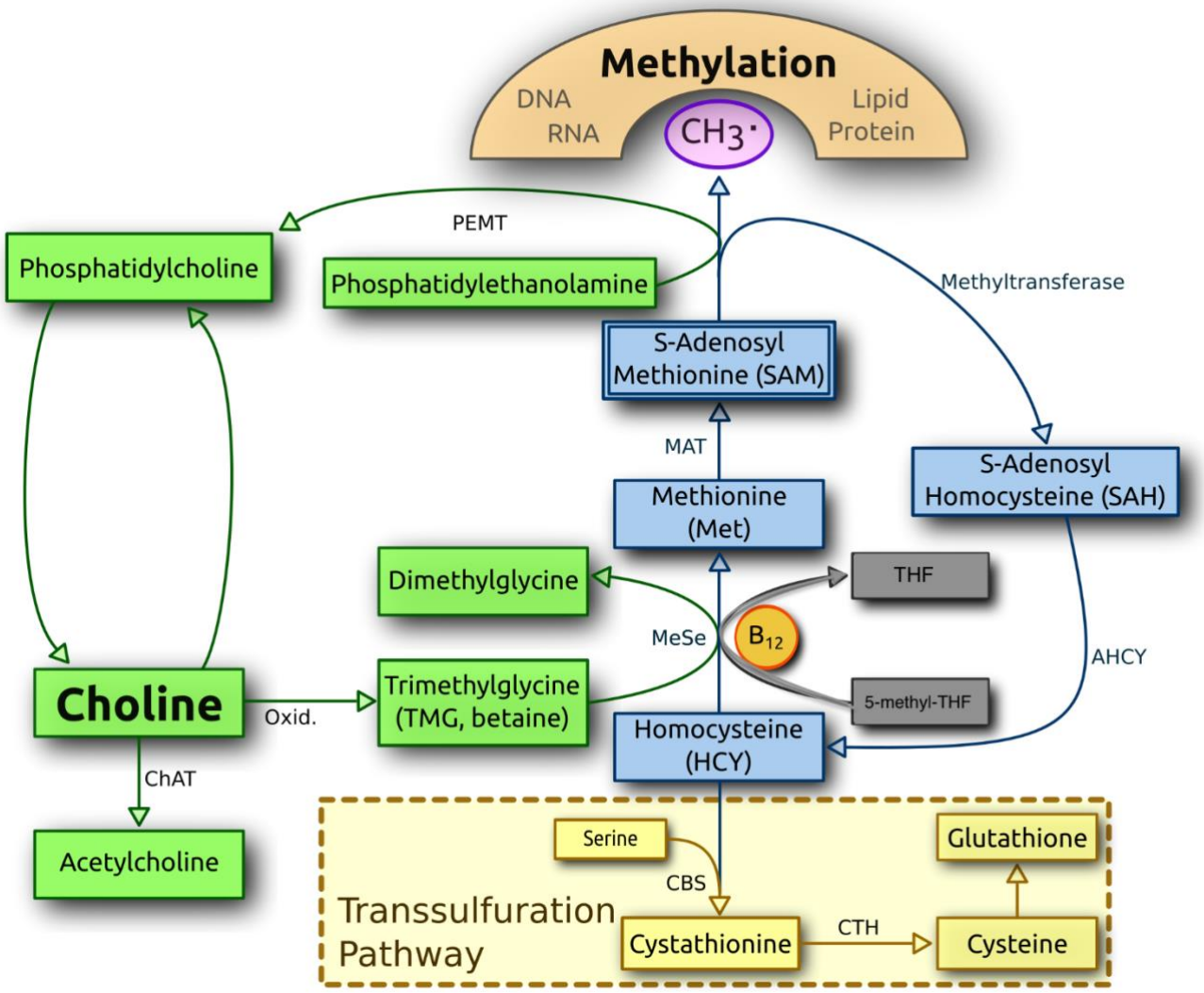

Figure 8. **An overview of homocysteine metabolism and the transsulfuration pathway**. CBS= cystathione-beta synthase; MeSe= methionine synthase; THF= tetrahydrofolate. (Figure uploaded by radio89 and labeled for reuse. https://commons.wikimedia.org/wiki/File:Choline_metabolism-en.svg. Image modified to present all terms in English.)

Serum methionine concentrations have been implicated in the outcomes of many long-term health studies in a vast selection of organisms [166]. It has been shown that lower consumption and subsequent blood concentrations of this essential amino acid is associated with longevity across species [166,167], as well as improved blood glucose tolerance in rats, lower levels of oxidative stress in mice [168], and a lower risk of developing cancers in both species [169,170]. The amount of food that dogs are fed may also affect dog health, however this consideration falls beyond the scope of the present study. Elevated serum methionine concentration serves as an indicator of overfeeding as has been shown in mice [171]. As there was considerably more meat-based protein present in the RMBD, it could be expected to be

reflected as higher blood serum and urine concentrations of methionine in the RMBD cohort. However, the KD manufacturer apparently adds an unspecified amount of DL-methionine to the kibble (Table S1a), which may in part explain this observation. Another explanation may be that canines in the KD cohort are actively eliminating or recycling greater concentrations of homocysteine than dogs fed the RMBD. In only the atopic dogs of the KD (n=6) cohort, there is a trend of higher homocysteine concentrations versus the atopic dogs of the RMBD (n=8) cohort (Supplementary file 21, sheet 23). Concurrently, there is also a trend of higher urine homocysteine concentrations of CAD-diagnosed KD-fed dogs from batch 1 (p=0.05714, FDR=0.1934) (Supplementary file 21, sheet 24). Although insignificant there is a trend of higher homocysteine concentrations in both urine and in the Batch 1 KD cohort (Supplementary file 21, sheets 19 and 24). In a previously reported study regarding the hematology of the canines during the diet intervention [3], it was determined that the canines in the KD fed cohort had elevated concentrations of blood serum B12 values. The significantly higher concentrations of methionine in the blood sera and urine of the batch 1 KD cohort (Table 3, Table S6), and concurrently higher B12 serum concentrations [3] may be partially due to increased methionine synthase activity [165] as homocysteine is converted to methionine via this pathway (Figure 7). The higher concentrations of B12 comports with a higher methionine/homocysteine ratio as methylated B12 is converted into B12, i.e. as its methyl group is donated to homocysteine, turning it into methionine. In the data reported by Anturaniemi et al. [3], serum folate concentrations were also significantly higher in KD-fed dogs, which also plays a role in homocysteine clearance.[165] In the present study however, concentrations of folic acid, the acid form of folate, were not significantly different between diet cohorts for either urine or serum. There is a correlation between the amount of B12 in the food with serum B12 in dogs[172], indicating that B12 concentrations in dogs are tightly regulated, i.e. conserved in dogs fed a diet low B12. Furthermore, 4-pyridoxic acid, a downstream product of pyridine (B6), was also found in significantly higher concentrations in both the serum and urine of the KD cohorts. As B6 is the cofactor for cystathione beta-synthase, which converts homocysteine to cystathionine via the transsulfuration pathway[173] (Figure 7), this may indicate that this pathway is significantly upregulated in the KD diet. Cystathionine, the first metabolite produced as a result of homocysteine clearance via the transsulfuration pathway[165] was found in far higher concentrations in all dogs in the KD cohort, with a high fold-change difference compared to the RMBD cohort (Table 3). Finally, higher serum concentrations of dimethylglycine were observed in all dogs in the KD cohort (Table 3), and a trend of higher serum concentration of betaine was found in all dogs in the RMBD cohort (Table S5), as well as in the urine of the batch 1 RMBD cohort (Table S6) compared to the KD cohort. Playing important roles in one-carbon metabolism, and subsequently often discussed in the context of DNA methylation,

betaine is converted to dimethylglycine as its methyl group is added to homocysteine, producing methionine (Figure 7).[174]

Elevated homocysteine levels are often discussed as risk factors for various canine pathologies, including cardiovascular disease,[175] increased inflammation,[176] and certain renal pathologies.[177] In humans, elevated levels of plasma homocysteine have been associated with irritable bowel syndrome and cancer.[178] Elevated homocysteine levels and subsequent clearance have long been known to be a risk marker for MetS in humans.[179] To our knowledge, no studies have observed any direct correlation between atopy and elevated homocysteine or methionine blood serum concentrations. However, a higher prevalence of atopic dermatitis in offspring was observed in the offspring of women with elevated circulating levels of vitamins B12 and folate, and hence upregulation of the homocysteine pathway may be related.[180] Homocysteine is highly toxic for dogs,[181] and blood homocysteine concentrations are kept low, lying within a narrow concentration range.[182] Studies on mice have shown that homocysteine concentrations are kept low even in the case where serum concentrations of methionine[171] as well as cystathionine[177] are significantly increased. We find a similar phenomenon in the present study. It should be noted that the blood homocysteine concentrations in the canines of both diet cohorts were no higher than those reported for healthy canines elsewhere.[183,184] The significantly higher blood serum and urine concentrations may indicate that more methionine was added to the diet than biologically necessary.[72,185] This may also be true of other metabolites found in significantly higher concentrations in both the serum and urine samples of batch 1 (Table 4a, Figure S2a), including 4-pyridoxic acid, which as discussed above is likely related to the significantly higher cystathionine concentrations observed in the KD cohort.

The atopic complex is still not fully understood in canines,[102] nor its relationship to MetS. Previous studies in mice[186] and humans[187] have provided contradictory evidence, indicating that AD both may[186] or may not[187] be linked to MetS in mammals. Whether underlying lifestyle choices predispose risk for both MetS and AD, or whether the development of MetS increases the risk of developing AD or vice versa, is not fully understood.[107] According to the evaluation of CAD severity at the end of the diet intervention, neither the KD nor the RMBD significantly changed the CADESI-4 score outcome of the CAD-diagnosed canines, although there was a trend of greater CADESI-4 worsening in the KD cohort (p=0.219) (Appendix table S3c). There was a general trend in worsening of CADESI-4 scores found in both diet cohorts (for the RMBD=6.9, σ=6.5, for the KD, μ=18.3, σ=13.8) (Appendix S3d and S3e, respectively). In order to avoid interference from the seasonality of the disease, the diet trial was originally planned to take place during the late fall and winter months, when plant allergens known to

exacerbate symptoms were not present. As discussed above, the trial had to be pushed forward, such that it ended when many plants had begun to bloom in Finland. It is likely that this delay caused the worsening of symptoms in both diet cohorts. There were disagreements between the owner-reported CAD diagnosis, which used the visual analogue scale, and the dermatologist's diagnosis, which used the CADESI-4 scale. A metabolomics approach can potentially address and classify differing phenotypes of CAD, by combining 'omics' with clinical and epidemiological data. However, in the present study when considering the targeted metabolomic analysis that compared the atopic and healthy individuals, there were no significantly different metabolite concentrations at either the baseline or the end of the diet trial (Figure 4a). This suggests that diagnosing CAD by studying the blood serum with the targeted metabolites used in this study is also challenging.

A couple of studies looking at macronutrient preference among dogs served several food choices of varying macronutrient compositions ad libitum have indicated that several breeds of dogs are well attuned to what they prefer and what their bodies require.[98,188] In the first study, the authors observed that several breeds of dogs adjusted to a preferred PFC macronutrient composition of 30:63:7% ME over a 7-day period,[188] and another study observed that Harrier Hound dogs adjusted to a PFC macronutrient ratio of 44:52:4% ME.[98] The adequacy of diets for domesticated dogs, especially with regard to macronutrient composition, have been studied by comparing their diet with the diet of wolf (*C. lupus*) populations.[189] A meta-analysis of 41 studies that observed the wolf diet in Europe and North America concluded that the average wolf diet has a PFC of 54:45:1% ME.[189] With the lack of carbohydrate and relatively high protein content, it resembles the RMBD used in our study (Table 1). This macronutrient ratio also resembles the ratio that the dogs in the two ad libitum studies mentioned above preferred.[98] The ratio these breeds tend towards comports with current nutritional guidelines for dogs,[190] which classify proteins and fats as essential, and carbohydrates as non-essential. It remains unclear whether increased starch digestibility offers any advantage to dogs with regard to their healthspan, or whether the artificial selection for improved tolerance towards a starch-rich diet may outweigh the predisposition for other illnesses. Both of these topics deserve further study.

5.2 Strengths and limitations of the study

5.2.1 Strengths and limitations of the study design

To our best knowledge this pilot study was the first ever to apply a serum and urine metabolomics-based approach to study how feeding canines a high-fat, moderate-protein, very low-carbohydrate RMBD affects serum and urine metabolite concentrations, as well as compare the outcome with the serum and urine metabolite profiles of dogs fed a moderate-fat, moderate-protein, high-carbohydrate KD. This targeted metabolomics approach offers quantitative and reliable data of blood serum and urine metabolite concentrations. Both urine and serum were analyzed simultaneously, giving insight into the relationships between the serum and urine media and diet. All dogs were pedigreed Staffordshire Bull Terriers. Their health status was diagnosed by a dermatologist using Favrot's criteria and the CADESI-4 scale to produce validated clinical scores.

As the present study focuses specifically on nutrition, there were no controls for quantitative markers for sleep, physical activity, or overall stress. Due to the high cost of analysis, the number of dogs that were used for the study were kept to a minimum of three dogs per cohort (KD-healthy, RMBD-healthy). As discussed in the Design and Animals section the postponed end of the diet intervention possibly allowed the introduction of undesired seasonal effects on CAD severity due to plant allergens. The study used more CAD-diagnosed than healthy dogs. Several dogs considered healthy prior to their official diagnosis by the dermatologist had to be reclassified as CAD-sufferers. There were no metabolites that significantly differed between diet cohorts of the healthy individuals at the end of the diet intervention (KD-healthy n=3, RMBD-healthy n=3). This is likely due to the small sample size. The far fewer significant differences in metabolites between diet cohorts of batch 2 (Table S8) may indicate that the underlying health status (CAD or healthy) had an impact on the results and may explain why the response to diet in the fully atopic cohort (batch 1) showed starker differences than for batch 2. Alternatively, this result may be an artifact due solely to the smaller sample size of batch 1.

5.2.2 Strengths and limitations of the instrumentation and analytical methods

The use of a UPLC-MS/MS platform for the targeted analysis of serum and urine metabolites has notable advantages over alternative approaches, many of which have been summarized in a recent review that compares various techniques for metabolomics-based analyses of biofluids.[27] Both the notable advantages and disadvantages of LC-MS over NMR and GC-MS approaches typically context-dependent. As the approach used in the present study focused on non-ionic compounds, polar

compounds, ESI was the most suitable option for the concentration range that most of the compounds were found in, however ion suppression due to matrix effects of the eluent while using ESI may explain the poor chromatographic data collected for certain compounds, especially spermidine. Furthermore many samples contained targeted compounds that were close to or below the lower level of quantification, indicating that the sensitivity of the MS instrument could benefit from more sensitive ion monitoring approaches, such as orbitrap, given that it would be important to study more compounds that may be found in even lower blood serum and urine concentrations in future studies. However, for this reason, it would be implausible to suggest the use of any approach other than a UPLC-MS/MS-style approach for producing quantifiable metabolite data.

Targeted metabolomic analysis of the serum samples collected from the dogs was performed in two batches. The ACQUITY UPLC/MS-MS instrument used for metabolomic analysis was serviced in between the analysis of the two batches, resulting in significantly different metabolite values between batches. Of the 102 metabolites targeted, a considerable amount had to be removed from the first batch analysis. Targeted analysis of the serum samples of the second batch went considerably better. Even so many of the metabolites were unable to be used in the combined batch analysis. The use of commercial IS kits helped save costs and generated quantifiable results, but it also caused us to focus on only a fraction of all metabolites in the samples studied, leaving the vast majority of metabolic data ignored. Given the vast variety of metabolites circulating in both serum and urine media, it is clear in retrospect that numerous metabolites not studied were worthy of analysis.

6 Conclusions

Three key differences were observed with regard to the effects of diet on the canine metabolite profiles studied. First, there were markedly higher levels of carnitines and related compounds in canines fed the RMBD. Additionally higher levels of nitrogen excretion were indicated, also a result of the diet's high meat content. Second, the KD-fed cohort showed elevated bile acid concentrations which have a condition implicated for example in colon tumorigenesis in mice and humans. In addition to reflecting the macronutrient profile it may also implicate a change in the gut microbiota composition. Further study is needed to confirm this. Third, there were higher concentrations of sulfur-containing compounds such as methionine and cystathionine, as well as compounds related to their metabolism, in the serum and urine of KD-fed dogs. Higher serum concentrations of these compounds are associated with increased inflammation in mammals. Furthermore, lower serum methionine concentrations as seen in the RMBD cohort, has long been established as a marker associated with long lifespan, and is

generally considered beneficial for metabolic health. The latter two differences suggest that the KD may be less beneficial to the metabolic health of canines as metabolite concentrations that have been previously implicated in various pathologies were found in higher concentrations than in the RMBD-fed dogs. Given the limitations of the present study however, such speculation requires further study to establish causality. Given the challenge of identifying CAD at the serum metabolite level, addressing and classifying differing phenotypes of CAD may be beyond the scope of a targeted metabolomics approach. Future studies will likely require both a larger set of metabolites to be targeted and larger sample cohorts. In summary, this experiment sought to clarify how nutrition may relate to CAD, as well as determine whether the impact of different diets could be seen on the metabolite level. While these topics are still novel for canine studies, the use of diet as a form of health maintenance, a notion that has gained popularity in recent years, will eventually be substantiated or rejected with quantitative clinical data.

7 Ethics Statement

Owners provided informed written consent for inclusion of their dogs in the study. The protocol was also approved by the Animal Experiment Board in Finland (ELLA) (permit number: ESAVI/3244/04.10.07/2013).

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9 Appendix

9.1 Tables

Table S1: An overview of the nutrient compostion of the diets used for the diet intervention

a) Nutrient composition of the KD used in the study. Values were provided by the manufacturer.

Hill's Science PlanTM Canine adult sensitive skin with chicken		
Composition: chicken (minimum chicken 23%, chicken and turkey combined 31%), ground rice, ground maize, chicken and turkey meal, maize gluten meal, dried whole egg, vegetable oil, flaxseed, digest, animal fat, potassium chloride, DL-methionine, salt, L-lysine hydrochloride, L-tryptophan, vitamins and trace elements. Naturally preserved with mixed tocopherols, citric acid and rosemary extract.		
Analytical Constituent	In Food	In Dry Matter
Protein (%)	25.3	27.5
Fat (%)	16	17.4
Carbohydrate (NFE) (%)	44.5	48.4
Fiber (crude) (%)	1.3	1.4
Ash (%)	4.9	5.3
Moisture (%)	8	
Calcium (%)	0.66	0.72
Phosphorus (%)	0.58	0.63
Calcium : Phosphorus	1.1	1.1
Sodium (%)	0.35	0.38
Potassium (%)	0.64	0.70
Magnesium (%)	0.07	0.08
Omega-3 fatty acids (%)	1.2	1.30
Omega-6 fatty acids (%)	4.8	5.22
ADDED per kg:		
Vitamin A (IU)	9600	10434.8
Vitamin D (IU)	480	522
Vitamin E (mg)	600	652
Vitamin C (mg)	70	76.1

Iron (mg)	53.7	58.4
Iodine (mg)	0.9	1.0
Copper (mg)	5.3	5.8
Manganese (mg)	5.6	6.1
Zinc (mg)	111	121
Selenium (mg)	0.15	0.2
Beta-carotene (mg)	1.5	1.6

b) Nutrient composition of the two RMBDs used in the study. Values were provided by the manufacturer

MUSH BARF Vaisto® diets					
Composition (pork-chicken-lamb): Finnish pork 46% (meat, lung, cartilage, heart, liver), Finnish chicken 29% (meat, bone, gizzard, skin, heart, cartilage, liver), Finnish lamb 20% (bone, meat, lung, cartilage, liver), vegetables 5% (spinach, broccoli, lettuce, cold-pressed sunflower oil), egg < 1%.			Composition (beef-turkey-salmon): Finnish beef, 47% (rumen, meat, lung, heart, cartilage, liver), Finnish turkey 38% (meat, bone, cartilage), Norwegian salmon 10% (salmon including bones), vegetables 5% (broccoli, lettuce, apple, carrot, cold-pressed sunflower oil, camelina oil).		
Analytical Constituent (pork-chicken-lamb)	In Food	In Dry Matter	Analytical Constituent	In Food	In Dry Matter
Protein (%)	15.2	38	Protein (%)	15	42.5
Fat (%)	20	50	Fat (%)	15.8	44.8
Fiber (crude) (%)	0.6	1.5	Fiber (crude) (%)	0.8	2.3
Ash (%)	4.2	10.5	Ash (%)	3.7	10.5
Moisture (%)	60	0	Moisture (%)	64.7	0
Calcium (%)	1.09	2.7	Calcium (%)	0.45	1.3
Phosphorus (%)	0.65	1.6	Phosphorus (%)	0.34	1.0
Calcium : Phosphorus	1.7	1.7	Calcium : Phosphorus	1.3	1.3
Analysed ingredients from different batch per kg			Analysed ingredients from different batch per kg		
Omega-3 fatty acids (%)		0.4	Omega-3 fatty acids (%)		1.1
Omega-6 fatty acids (%)		3.8	Omega-6 fatty acids (%)		2.7

Vitamin A (IU)		143050	Vitamin A (IU)		80890
Vitamin D (IU)		698	Vitamin D (IU)		2130
Vitamin E (mg)		46.6	Vitamin E (mg)		54.4
Vitamin C (mg)		123	Vitamin C (mg)		82.1
Iodine (mg)		1.86	Iodine (mg)		1.64
Copper (mg)		24.2	Copper (mg)		31.5
Manganese (mg)		8.8	Manganese (mg)		7.4
Zinc (mg)		119	Zinc (mg)		79.6
Selenium (mg)		0.62	Selenium (mg)		0.73

Table S2a-c: Overview of metabolites that were included and removed prior to data analysis, with reason's for removal.

a) Batch 1 (*n=8*) Serum

Removed metabolites (*n=22*)	Reason(s) for removal	Included metabolites (*n=80*)			
Leucine	ISTD[1]	Glycine	Creatine	Kynurenine	g-Glu-Cy
Hypoxanthine	ISTD	Trimethylamine-N-Oxide	Aspartate	Pantothenic Acid	Inosine
Carnitine	ISTD	Alanine	Adenine	Cystathionine	Guanosine
Cytidine	ISTD	Glyceraldehyde	Homocysteine	3-Hydroxykynurenine	cGMP
Isobutyrylcarnitine	ISTD	GABA	Spermidine	2-deoxycytidine	IMP
Phosphoethanolamine	Poor Chromatography	Dimethyl Glycine	Lysine	Carnosine	Glycocholic Acid
AMP	Poor Chromatography	Aminoisobutyric acid	Glutamine	Adenosine	Taurochenodeoxycholic Acid
3-OH-Anthanilic Acid	Poor Chromatography	Choline	Glutamic Acid	Xanthosine	Taurocholic Acid
Sorbitol	Poor Chromatography	Serine	Methionine	Glutathione (reduced)	Acetylcarnitine
2-deoxyuridine	Poor Chromatography	Cytosine	Histidine	Folic Acid	Propionylcarnitine
Sucrose	Poor Chromatography	Creatinine	Normetanephrine	Uracil	Isovalerylcarnitine
Chenodeoxycholic Acid	Poor Chromatography	Proline	Phenylalanine	Succinate	Hexanoylcarnitine
UDP-Glucose	Poor Chromatography	Betaine	Arginine	Homoserine	Octanoylcarnitine

Cholic Acid	Missing values (>50%)	Valine	Citrulline	Threonine	Decanoylcarnitine
NAD	Missing values (>50%)	Guanidinoacetic Acid	Hippuric acid	Nicotinic Acid	
Acetoacetic acid	Missing values (>50%)	Niacinamide (B3)	Tyrosine	Xanthine	
Homogentisic acid	Missing values (>50%)	Taurine	4-Pyridoxic Acid	Orotic acid	
Pyridoxine (B6)	Missing values (>50%)	1-methylhistamine	Kynurenic Acid	Allantoin	
Cotinine	Missing values (>50%)	Isoleucine	5-Hydroxyindole-3-acetic acid	2-Aminodipic Acid	
L-5-Hydroxytryptophan	Missing values (>50%)	Hydroxyproline	SDMA	Inositol	
Neopterin	Missing values (>50%)	Asparagine	ADMA	Glucoronate	
cAMP	Missing values (>50%)	Ornithine	Tryptophan	Ribose-5-P	

[1]ISTD= internals standard discrepancy/ calibration error

b) Batch 1 (*n=8*) Urine)

Removed metabolites (*n=22*)	Reason(s) for removal	Included metabolites (*n=80*)			
Leucine	ISTD[1]	Glycine	Creatine	Kynurenine	g-Glu-Cy
Hypoxanthine	ISTD	Trimethylamine-N-Oxide	Aspartate	Pantothenic Acid	Inosine
Carnitine	ISTD	Alanine	Adenine	Cystathionine	Guanosine
Cytidine	ISTD	Glyceraldehyde	Homocysteine	3-Hydroxykynurenine	cGMP
Isobutyrylcarnitine	ISTD	GABA	Spermidine	2-deoxycytidine	IMP
Phosphoethanolamine	Poor Chromatography	Dimethyl Glycine	Lysine	Carnosine	Glycocholic Acid
AMP	Poor Chromatography	Aminoisobutyric acid	Glutamine	Adenosine	Taurochenodeoxycholic Acid
3-OH-Anthanilic Acid	Poor Chromatography	Choline	Glutamic Acid	Xanthosine	Taurocholic Acid
Sorbitol	Poor Chromatography	Serine	Methionine	Glutathione (reduced)	Acetylcarnitine
2-deoxyuridine	Poor Chromatography	Cytosine	Histidine	Folic Acid	Propionylcarnitine

Sucrose	Poor Chromatography	Creatinine	Normetanephrine	Uracil	Isovalerylcarnitine
Chenodeoxycholic Acid	Poor Chromatography	Proline	Phenylalanine	Succinate	Hexanoylcarnitine
UDP-Glucose	Poor Chromatography	Betaine	Arginine	Homoserine	Octanoylcarnitine
Cholic Acid	Missing values (>50%)	Valine	Citrulline	Threonine	Decanoylcarnitine
NAD	Missing values (>50%)	Guanidinoacetic Acid	Hippuric acid	Nicotinic Acid	
Acetoacetic acid	Missing values (>50%)	Niacinamide (B3)	Tyrosine	Xanthine	
Homogentisic acid	Missing values (>50%)	Taurine	4-Pyridoxic Acid	Orotic acid	
Pyridoxine (B6)	Missing values (>50%)	1-methylhistamine	Kynurenic Acid	Allantoin	
Cotinine	Missing values (>50%)	Isoleucine	5-Hydroxyindole-3-acetic acid	2-Aminodipic Acid	
L-5-Hydroxytryptophan	Missing values (>50%)	Hydroxyproline	SDMA	Inositol	
Neopterin	Missing values (>50%)	Asparagine	ADMA	Glucoronate	
cAMP	Missing values (>50%)	Ornithine	Tryptophan	Ribose-5-P	

[1]ISTD= internals standard discrepancy/ calibration error

c) Batch 1 & 2 combined (All dogs, *n=20*) Serum

Removed metabolites (*n = 23*)	Reason(s) for removal	Included metabolites (*n = 79*)			
Leucine	ISTD[1]	Glycine	Aspartate	Cystathionine	cGMP
Hypoxanthine	ISTD	Trimethylamine-N-Oxide	Adenine	3-Hydroxykynurenine	IMP
Carnitine	ISTD	Alanine	Homocysteine	2-deoxycytidine	Glycocholic Acid
Cytidine	ISTD	Glyceraldehyde	Spermidine	Carnosine	Taurochenodeoxycholic Acid
Isobutyrylcarnitine	ISTD	GABA	Lysine	Adenosine	Taurocholic Acid
Spermidine	Poor Chromatography, CC, ISTD	Dimethyl Glycine	Glutamine	Xanthosine	Acetylcarnitine
Phosphoethanolamine	Poor Chromatography	Aminoisobutyric acid	Glutamic Acid	Glutathione (reduced)	Propionylcarnitine

AMP	Poor Chromatography	Choline	Methionine	Folic Acid	Isovalerylcarnitine
3-OH-Anthanilic Acid	Poor Chromatography	Serine	Histidine	Uracil	Hexanoylcarnitine
Sorbitol	Poor Chromatography	Cytosine	Normetanephrine	Succinate	Octanoylcarnitine
2-deoxyuridine	Poor Chromatography	Creatinine	Phenylalanine	Homoserine	Decanoylcarnitine
Sucrose	Poor Chromatography	Proline	Arginine	Threonine	
Chenodeoxycholic Acid	Poor Chromatography	Betaine	Citrulline	Nicotinic Acid	
UDP-Glucose	Poor Chromatography	Valine	Hippuric acid	Xanthine	
Cholic Acid	Missing values (>50%)	Guanidinoacetic Acid	Tyrosine	Orotic acid	
NAD	Missing values (>50%)	Niacinamide (B3)	4-Pyridoxic Acid	Allantoin	
Acetoacetic acid	Missing values (>50%)	Taurine	Kynurenic Acid	2-Aminodipic Acid	
Homogentisic acid	Missing values (>50%)	1-methylhistamine	5-Hydroxyindole-3-acetic acid	Inositol	
Pyridoxine (B6)	Missing values (>50%)	Isoleucine	SDMA	Glucoronate	
Cotinine	Missing values (>50%)	Hydroxyproline	ADMA	Ribose-5-P	
L-5-Hydroxytryptophan	Missing values (>50%)	Asparagine	Tryptophan	g-Glu-Cy	
Neopterin	Missing values (>50%)	Ornithine	Kynurenine	Inosine	
cAMP	Missing values (>50%)	Creatine	Pantothenic Acid	Guanosine	

[1]ISTD= internals standard discrepancy/ calibration error

Table S3a-e: An overview of the relationship between diet cohorts as well as at end of diet intervention with the change in CADESI scores, weight change, age of cohorts

a) CADESI score, age, and weight change in relation to diet of all dogs (n=20)

Variable	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
CADESI[5] change	16.889 (11.152)	9.364 (7.103)	0.1586	0.3515	1.8	Up
Age (months)	74.111 (37.926)	62.000 (34.351)	0.4636	0.6233	1.2	Up
Weight change, kg	0.434 (0.721)	-0.154 (1.047)	0.1709	0.3687	0.36	Up

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate; [5]CADESI= Canine Atopic Dermatitis Extent and Severity Index

b) CADESI score, age, and weight change in relation to diet of healthy dogs (n=6)

Name	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
CADESI[5] change	14.000 (1.732)	13.333 (5.859)	0.6579	0.8701	1.05	Up
Age (months)	97.000 (57.611)	96.667 (29.939)	0.9933	1	1	Up
Weight change, kg	0.267 (0.550)	-0.766 (1.243)	0.2583	0.8029	2.87	Up

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate; [5]CADESI= Canine Atopic Dermatitis Extent and Severity Index

c) CADESI score, age, and weight change in relation to diet of CAD-diagnosed dogs (n=14)

Name	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
CADESI[5] change	18.333 (13.794)	7.875 (7.279)	0.2185	0.437	2.33	Up
Age (months)	62.667 (22.411)	49.000 (26.859)	0.3333	0.5941	1.28	Up
Weight change, kg	0.517 (0.828)	0.076 (0.951)	0.3837	0.6409	6.78	Up

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate; [5]CADESI= Canine Atopic Dermatitis Extent and Severity Index

d) CADESI score, age, and weight change in relation to health status of RMBD-fed dogs (n=11)

Name	Mean (SD[1]) of CAD[2]	Mean (SD) of H[3]	p-value	q-value (FDR[4])	Fold Change	In CAD-diagnosed cohort
CADESI[5] change	7.875 (7.279)	13.333 (5.859)	0.2783	0.8453	-1.69	Down
Age (months)	49.000 (26.859)	96.667 (29.939)	0.031*	0.5087	-1.97	Down
Weight change, kg	0.076 (0.951)	-0.763 (1.245)	0.2566	0.835	10.02	Up

[1]SD= Standard deviation; [2]CAD= Canine Atopic Dermatitis; [3]H= Healthy; [4]FDR= False Discovery Rate; [5]CADESI= Canine Atopic Dermatitis Extent and Severity Index

e) CADESI score, age, and weight change in relation to health status of KD-fed dogs (n=9)

Name	Mean (SD[1]) of CAD[2]	Mean (SD) of H[3]	p-value	q-value (FDR[4])	Fold Change	In CAD-diagnosed cohort
CADESI[5] change	18.333 (13.794)	14.000 (1.732)	0.6025	0.8892	1.31	Up
Age (months)	62.667 (22.411)	97.000 (57.611)	0.2212	0.7856	-1.55	Down
Weight change kg	0.517 (0.828)	0.267 (0.550)	0.6563	0.8892	1.93	Up

[1]SD= Standard deviation; [2]CAD= Canine Atopic Dermatitis; [3]H= Healthy; [4]FDR= False Discovery Rate; [5]CADESI= Canine Atopic Dermatitis Extent and Severity Index

Table S4: GLM comparing serum metabolite concentrations of diet cohorts at baseline of diet intervention (n=20)
Metabolite concentrations that differed at baseline of diet intervention between the diet cohorts ($p<0.05$, FDR>0.05) (total n=20). *NB* that the canines' diets prior to baseline were not controlled for.

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Arginine	7.229 (0.266)	7.551 (0.203)	0.0066	0.5256	-1.04	Down
Histidine	6.224 (0.182)	6.421 (0.142)	0.0139	0.5548	-1.03	Down
Threonine	7.764 (0.489)	8.151 (0.319)	0.0472	0.749	-1.05	Down

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S5: GLM of all dogs comparing serum metabolite concentrations of diet cohorts at end of intervention

Comparison of all individuals (n=20) between diet cohorts at end of diet intervention, including all metabolites below p<0.05

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Methionine	6.686 (0.294)	5.697 (0.305)	< 0.0001	0	1.17	Up
4-Pyridoxic Acid	-8.830 (0.460)	-11.025 (0.804)	< 0.0001	0	-1.25	Up
Citrulline	5.659 (0.204)	4.654 (0.507)	< 0.0001	0.0011	1.22	Up
Cytosine	-4.146 (0.790)	-5.964 (0.930)	0.0002	0.0026	-1.44	Up
Proline	7.965 (0.406)	7.099 (0.403)	0.0002	0.0026	1.12	Up
Cystathionine	3.154 (1.292)	0.152 (1.004)	0.0002	0.0026	20.78	Up
Taurochenodeoxycholic Acid	-0.898 (0.762)	-3.255 (1.357)	0.0002	0.0026	-3.62	Up
Hexanoylcarnitine	-7.033 (0.484)	-5.937 (0.760)	0.0015	0.0148	1.18	Down
Decanoylcarnitine	-6.414 (0.485)	-5.443 (0.661)	0.0018	0.0156	1.18	Down
Glycine	8.629 (0.299)	8.049 (0.407)	0.0023	0.018	1.07	Up
Creatine	4.155 (0.616)	5.176 (0.753)	0.0043	0.0297	-1.25	Down
Kynurenine	0.849 (0.513)	0.242 (0.319)	0.0045	0.0297	3.51	Up
Dimethylglycine	2.369 (0.511)	1.606 (0.575)	0.0062	0.0374	1.48	Up
Trimethylamine-N-Oxide	-3.100 (11.157)	1.534 (0.830)	0.0074	0.042	0.49	Down
IMP	-6.609 (7.565)	-2.445 (1.389)	0.0097	0.051	2.7	Down
Octanoylcarnitine	-6.080 (0.410)	-5.312 (0.713)	0.0104	0.0515	1.14	Down
2-Aminoisobutyric acid	-1.810 (0.218)	-2.219 (0.419)	0.0165	0.0769	-1.23	Up
Betaine	6.766 (0.327)	7.228 (0.463)	0.0200	0.0861	-1.07	Down
Acetylcarnitine	1.619 (0.520)	2.170 (0.452)	0.0207	0.0861	-1.34	Down
Taurocholic Acid	-0.940 (0.717)	-1.756 (0.795)	0.0283	0.1117	-1.87	Up
Tryptophan	5.359 (0.361)	5.025 (0.284)	0.0323	0.1215	1.07	Up
Creatinine	6.842 (0.225)	7.050 (0.200)	0.0414	0.1457	-1.03	Down
Asparagine	6.518 (0.270)	6.163 (0.420)	0.0424	0.1457	1.06	Up
Aminoadipic acid	1.365 (0.589)	1.874 (0.478)	0.047	0.1547	-1.37	Down

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S6: GLM of batch 1 urine samples comparing diet cohorts at end of diet intervention

Batch 1 Urine (n=8) comparison of diet cohorts at end of diet intervention (FDR<0.05)

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Methionine	8.937 (0.771)	5.306 (0.529)	0.0002	0.0192	1.68	Up
Cytosine	5.321 (0.256)	3.254 (0.550)	0.0005	0.0196	1.63	Up
Betaine	9.200 (0.422)	10.665 (0.214)	0.0008	0.0219	-1.16	Down
4-Pyridoxic Acid	0.942 (0.219)	-0.737 (0.593)	0.0018	0.0364	0.78	Up
Creatine	8.307 (0.401)	11.074 (1.019)	0.0023	0.037	-1.33	Down
Isoleucine	6.913 (0.137)	6.413 (0.157)	0.003	0.037	1.08	Up
Hydroxyproline	5.523 (1.512)	9.547 (0.877)	0.0037	0.037	-1.73	Down
Uracil	1.700 (0.297)	3.930 (0.923)	0.0037	0.037	-2.31	Down
Homoserine	1.275 (0.190)	1.968 (0.267)	0.0055	0.049	-1.54	Down

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S7: GLM of Batch 1 dogs comparing serum metabolite concentrations between diet cohorts at end of diet intervention

Batch 1 (N=8) comparison of diet cohorts at end of diet intervention (FDR<0.05)

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Ribose-5-P	-0.150 (0.297)	1.001 (0.104)	0.0003	0.0204	6.67	Down
Citrulline	5.947 (0.284)	4.748 (0.239)	0.0006	0.0204	1.25	Up
Cystathionine	3.245 (0.913)	0.240 (0.374)	0.0009	0.0204	13.53	Up
Methionine	6.853 (0.268)	5.835 (0.215)	0.001	0.0204	1.17	Up
Cytosine	-3.596 (0.778)	-5.865 (0.516)	0.0028	0.0389	-1.63	Up
4-Pyridoxic Acid	-8.755 (0.502)	-10.678 (0.619)	0.0029	0.0389	-1.22	Up

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S8: GLM of Batch 2 dogs comparing serum metabolite concentrations between diet cohorts at end of diet intervention

Batch 2 (N=12) comparison of diet cohorts at end of diet intervention (FDR<0.05)

Metabolite	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Taurochenodeoxycholic Acid	-0.320 (0.534)	-3.603 (1.105)	0.0001	0.0121	-11.25	Up
4-Pyridoxic Acid	-8.840 (0.467)	-11.466 (1.014)	0.0003	0.0167	-1.3	Up
Methionine	6.585 (0.362)	5.552 (0.398)	0.001	0.0337	1.19	Up

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S9: GLM of CAD-diagnosed dogs comparing serum metabolite concentrations between diet cohorts at end of intervention

Comparison of all atopic individuals (n=14) between diet cohorts at end of diet intervention

Name	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Methionine	6.746 (0.310)	5.719 (0.288)	< 0.0001	0.0024	1.18	Up
Proline	8.056 (0.402)	7.096 (0.228)	0.0001	0.004	1.14	Up
Glycine	8.692 (0.269)	7.967 (0.252)	0.0002	0.0045	1.09	Up
Citrulline	5.701 (0.229)	4.653 (0.433)	0.0002	0.0045	1.23	Up
Dimethylglycine	2.584 (0.410)	1.555 (0.427)	0.0007	0.0076	1.66	Up
Cytosine	-3.961 (0.873)	-6.222 (0.943)	0.0006	0.0076	-1.57	Up
4-Pyridoxic Acid	-8.842 (0.445)	-11.101 (0.767)	0.0007	0.0076	-1.26	Up
Cystathionine	3.001 (1.484)	-0.057 (1.116)	0.0008	0.0083	0.02	Up
2-Aminoisobutyric acid	-1.780 (0.183)	-2.254 (0.250)	0.0021	0.0183	-1.27	Up
Creatine	4.136 (0.675)	5.403 (0.579)	0.0026	0.0209	-1.31	Down
Hexanoylcarnitine	-6.921 (0.485)	-5.765 (0.753)	0.0067	0.0483	1.2	Down
Decanoylcarnitine	-6.415 (0.595)	-5.330 (0.646)	0.0074	0.0488	1.2	Down

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S10: GLM of healthy dogs comparing serum metabolite concentrations of diet cohorts at end of diet intervention

Comparison of healthy individuals (n=6) between diet cohorts at end of diet intervention ($p<0.05$)

Name	Mean (SD[1]) of KD[2] cohort	Mean (SD) of RMBD[3] cohort	p-value	q-value (FDR[4])	Fold Change	In KD cohort
Decanoylcarnitine	-6.540 (0.164)	-5.239 (0.294)	0.0026	0.203	1.25	Down
Taurochenodeoxycholic Acid	-0.309 (0.429)	-3.669 (1.067)	0.0072	0.2836	-11.88	Up
Methionine	6.442 (0.122)	5.711 (0.342)	0.0253	0.4407	1.13	Up
Citrulline	5.629 (0.122)	4.961 (0.320)	0.0279	0.4407	1.13	Up
4-Pyridoxic Acid	-8.626 (0.334)	-10.599 (0.843)	0.0196	0.4407	-1.23	Up
Hexanoylcarnitine	-6.916 (0.366)	-5.682 (0.623)	0.0417	0.4752	1.22	Down
Octanoylcarnitine	-6.122 (0.303)	-5.104 (0.516)	0.0421	0.4752	1.2	Down

[1]SD= Standard deviation; [2]KD= Kibble Diet; [3]RMBD= Raw meat-based diet; [4]FDR= False Discovery Rate

Table S11: Overview of differences between urine and serum of diet cohorts

a) Metabolite concentrations are significantly (FDR<0.05) higher or lower in both urine and serum samples

Metabolite	Urine (FDR[1])	Serum (FDR)	In KD[2] cohort
Cytosine	0.0059327	0.0069354	Up
Methionine	0.0016249	0.0076431	Up
4-Pyridoxic Acid	0.013226	0.0069354	Up
Betaine	0.013226	0.047101	Down
Creatine	0.0059327	0.0069354	Down

[1]FDR= False Discovery Rate; [2]KD= Kibble Diet

b) Metabolite concentrations that significantly differ (FDR<0.05) between diet cohorts in either urine or serum, but not both

Metabolite	Urine (FDR[1])	Serum (FDR)	In KD[2] cohort
GABA	0.013226	0.93735	Up
Citrulline	0.77481	0.0069354	Up
Cystathionine	0.2709	0.0068914	Up
Alanine	0.0059327	0.85065	Down
Hydroxyproline	0.0059327	0.90683	Down
Adenine	0.033022	0.73449	Down
Folic Acid	0.032336	0.94688	Down
Uracil	0.012365	0.88737	Down
Ribose-5-P	0.31033	0.0069354	Down

[1]FDR= False Discovery Rate; [2]KD= Kibble Diet

Table S12: Two-way ANOVA of all dogs between health status and diet cohorts

Two-way ANOVA results from all dogs comparison between the RMBD and KD diet cohorts (RMBD= raw meat based diet, KD= kibble diet) RMBD) and health status (health and CAD).

Metabolite	Diet (F-value)	Diet (p-value)	Diet (FDR[1])	Health status (F-value)	Health status (p-value)	Health status (FDR)	Interaction (F-value)	Interaction (p-value)	Interaction (FDR)
Methionine	50.155	0.0000025959	0.00013647	0.71064	0.41166	0.81318	0.11327	0.74083	0.90344
4-Pyridoxic Acid	47.872	0.0000034549	0.00013647	0.22242	0.64357	0.86173	0.12286	0.73052	0.90344
Cystathionine	32.844	0.000030949	0.00081498	1.1789	0.29366	0.81318	0.072054	0.7918	0.90344
Citrulline	27.838	0.000075305	0.0014873	0.079115	0.78211	0.93694	0.098471	0.75773	0.90344
Cytosine	23.64	0.000173	0.0027334	0.31489	0.58247	0.81318	3.4045	0.08361	0.76625
Taurochenodeoxycholic Acid	22.711	0.00021058	0.0027727	0.099986	0.75593	0.93694	2.9489	0.10523	0.76625
Proline	21.233	0.00029094	0.0032835	0.37639	0.54816	0.81318	0.48891	0.49446	0.87327
Hexanoylcarnitine	14.495	0.0015488	0.015294	2.4372	0.13805	0.81318	0.22119	0.64448	0.90344
Decanoylcarnitine	12.697	0.0025931	0.020838	0.52946	0.47735	0.81318	0.50097	0.48926	0.87327
Glycine	12.639	0.0026377	0.020838	0.14097	0.71225	0.92242	1.9031	0.18671	0.86202
Creatine	11.475	0.0037579	0.026988	1.5408	0.2324	0.81318	1.8403	0.19375	0.86202
Dimethylglycine	10.295	0.0054788	0.034996	0.67286	0.42411	0.81318	2.5887	0.12718	0.76625
Kynurenine	10.143	0.0057588	0.034996	0.97885	0.33721	0.81318	0.37558	0.54859	0.87327

[1]FDR= False Discovery Rate

Table S13: Fisher's LSD comparison between CAD-RMBD (*n=8*), Healthy-RMBD (*n*=3), CAD-KD (*n=6*) cohorts and Healthy-KD cohorts (*n=3*)

Four-group analysis between the atopic (CAD) and healthy canines of both diet cohorts (RMBD= raw meat based diet, KD= kibble diet) using Fisher's LSD test showing the significant differences between cohorts. The results from this table are presented in figure S3.

Metabolite	F-value	p-value	FDR[1]
Methionine	16.993	0.000031415	0.0017278
4-Pyridoxic Acid	16.072	0.000043741	0.0017278
Cystathionine	11.365	0.00030588	0.0080548
Citrulline	9.3387	0.000837	0.014859
Cytosine	9.1199	0.00094046	0.014859
Taurochenodeoxycholic Acid	8.5868	0.0012584	0.016569
Proline	7.3663	0.0025557	0.028843

[1]FDR= False Discovery Rate

Table S14: PLS-DA model cross-validation scores. The accuracy, R2 and Q2 model parameters were determined by testing the PLS-DA model with the leave-one-out cross validation (LOOCV) method.

a) Accuracy, R2 and Q2 parameters for the 2 component PLS-DA model between sample media (serum and urine) and diet cohorts (raw meat-based and kibble) of Batch 1 (n=8) at the end of the diet intervention

Diet vs Media	
Measure	2 component model
Accuracy	0.5
R2	0.40314
Q2	0.10804

b) Accuracy, R2 and Q2 parameters for the 2 component PLS-DA model between health status cohorts (CAD and healthy) and diet cohorts (raw meat-based and kibble) of both batches (n=20) at the end of the diet intervention

Diet vs Health status	
Measure	2 component model
Accuracy	0.4
R2	0.586
Q2	0.277

9.2 Supplementary Figures

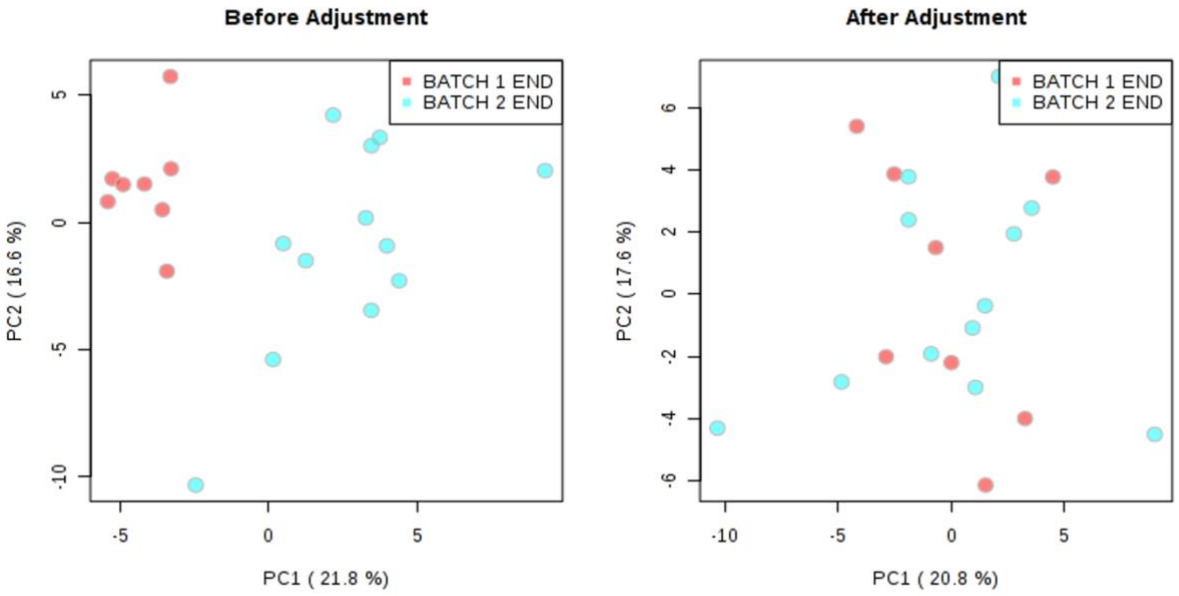

Figure S1- Batch correction combines the 2 batches used in the diet intervention

A PCA-plot showing the effect of the batch correction on the metabolite values of all dogs (*n=20*) (The end of the diet trial is shown here) using the ComBat method.

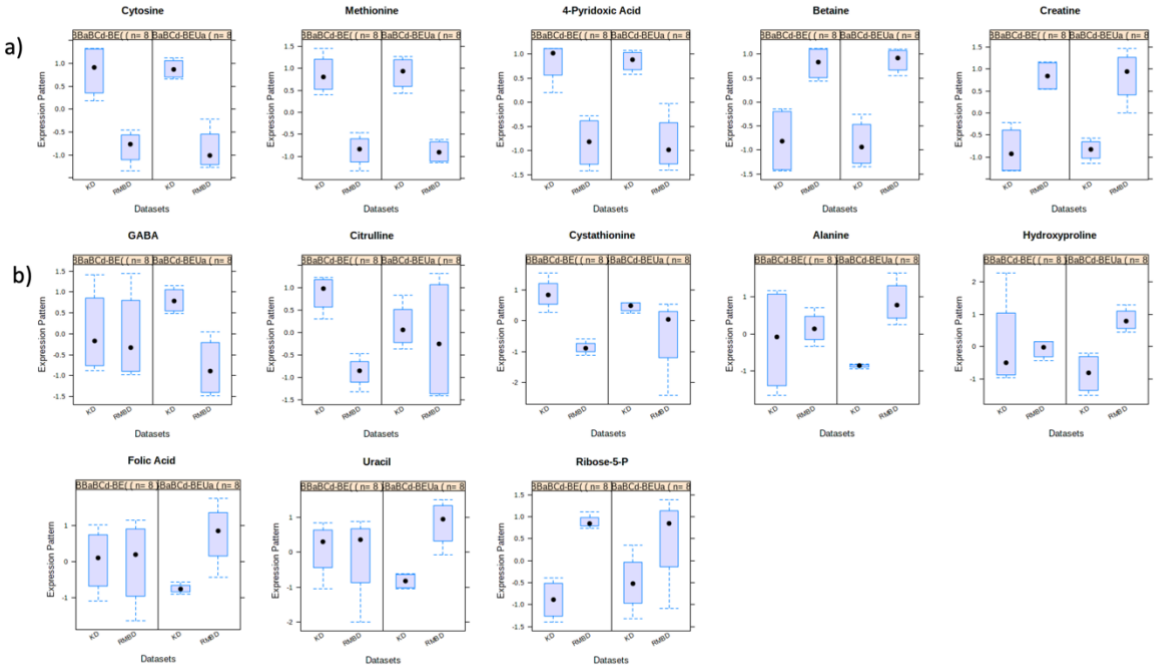

Figure S2 – Urine and serum meta-analysis comparison

a) Relative urine (left) and serum (right) concentrations of metabolites that correspondingly were either higher or lower in both urine and serum samples between diet cohorts (Table S10a).

b) Relative and serum (left) urine (right) concentrations of metabolites that correspondingly were significantly higher or lower in either urine and serum samples between diet cohorts (Table S10b).

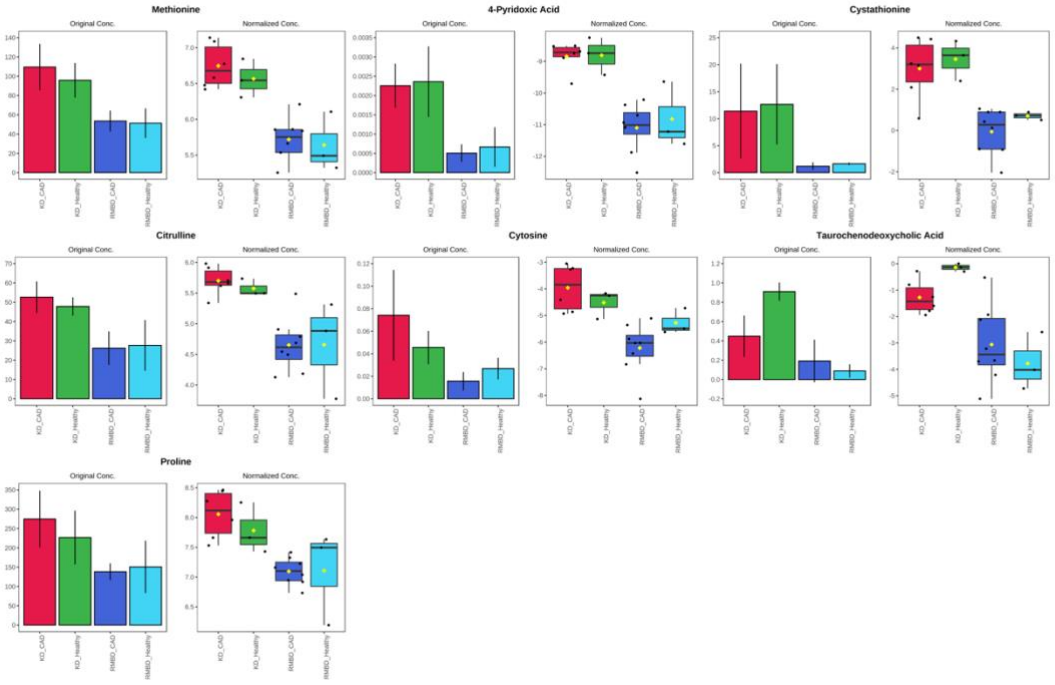

Figure S3

Original and log-transformed concentrations of metabolites that significantly differ between the four-group analysis using Fisher's LSD (Table S12)