Metabolomics: an alternative approach to canine epilepsy

Author : TSZ HONG LAW, **HOLGER VOLK**, **JON BOWEN**

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TSZ HONG LAW BSc, MRes

HOLGER VOLK DVM, PGCAP, DipECVN, PhD, FHEA, MRCVS

JON BOWEN BVetMed, DipAS(CABC), MRCVS

consider the use of high-end technology, specifically metabolomics, as an alternative approach to understanding epilepsy in dogs

EPILEPSY of unknown aetiology, a chronic neurological condition characterised by recurrent seizures, is thought to have a prevalence that ranges from 0.5 per cent to five per cent and may vary between species[1].

A study involving more than 500 dogs has shown a prevalence of 0.6 per cent in first opinion practice[1].

Epilepsy is naturally occurring in many different species, including goats, cattle, dogs, cats, rodents, non-human and human primates[2]. Canine epilepsy is similar in semiology to its human counterpart. Dogs live in the same environment as humans and are exposed to similar external factors.

Epilepsy is usually categorised into three subgroups known as idiopathic epilepsy, symptomatic epilepsy and cryptogenic (possible symptomatic) epilepsy.

Idiopathic epilepsy refers to epilepsy with an unknown cause and presumed to have a genetic basis[3], [4], [5]. Diagnosis of idiopathic epilepsy involves systematically eliminating possible causes of seizures until no underlying causes can be identified. Typical diagnosis usually involves a detailed neurological exam, haematology and biochemistry, dynamic bile acid, serum albumin, MRI and cerebral spinal fluid analysis[2].

Symptomatic epilepsy refers to epileptic seizures caused by a known or identified disorder with specific aetiologies, such as brain tumour, traumatic scar tissue, inflammation or infectious disease and congenital malformations[6].

Cryptogenic epilepsy refers to epilepsy that is suspected to be symptomatic; however, the exact aetiology remains undetermined.

Epileptic seizures are generally distinguished according to the origin of the seizure in the brain. Seizures that appear to be localised, where initial activation happens in one part of one cerebral hemisphere or a specific region of the fore-brain, are known as focal or partial seizures[7], [8], [9]. In humans, partial seizures can be further subdivided into either simple or complex partial seizures, where consciousness is either unimpaired or impaired, respectively[9], [10], [11]. Other seizures that arise from simultaneous activation of both cerebral hemispheres are known as generalised seizures[7], [8], [10]. In most cases observed in canine epilepsy, seizures are partial seizures with secondary generalisation. Here, seizures originate in a localised and specific area of the brain, but rapidly project to other areas or spread throughout the entire brain[6].

There are thought to be four phases to an epileptic seizure event, consisting of prodrome, aura, ictus and postictal phases. Identification of these phases can be useful when trying to differentiate a genuine epileptic seizure from other possible causes of seizure episodes, such as syncope, movement disorders or episodic neuromuscular weakness[2].

Prodrome refers to long-term indications, such as behavioural changes, that typically occur hours to days before an epileptic seizure. In humans, prodromal indicators may include unexpected emotional aberrations such as anxiety and irritability. In dogs, restlessness has been reported as the most common prodromal sign[5], [6].

The aura phase refers to short-term indications that typically occur a few seconds to a few minutes before seizures. In human epilepsy, aura that occurs on its own is recognised to be a sensory focal seizure, whereas in veterinary neurology the aura phase is only recognised as a preictal event[2].

The ictus phase refers to the actual epileptic seizure event and the postictal phase refers to abnormal behavioural aberrations hours to days after the seizure. Typical postictal phase clinical signs include ataxia, lethargy, hunger/thirst, loss of sight and aggressiveness[5], [6].

Despite ongoing research in understanding the pathophysiological manifestation of seizures and

epilepsy, the cellular mechanisms remain elusive. As a result, approaches towards epileptic therapy are directed towards the control of seizures; that is, chronic administration of anti-epileptic (anticonvulsant) drugs (AEDs), rather than prevention of epileptogenesis[3]. Attention has thus far been concentrated on developing better AEDs with higher anticonvulsant efficacy and less toxicity and minimal drug-induced side effects[13], [13].

Without a clear understanding or knowledge of the cellular processes involved in an epileptic seizure, identification of new AEDs have been restricted to either screening (serendipity) or structural variation development of existing drugs rather than by rational strategies[3].

Some of the antiepileptic medication routinely used in canine epilepsy include phenobarbital, potassium bromide, imepitoin, benzodiazepines, gabapentin and levetiracetam[6], [12], [14].

Although appropriate AED therapy results in a preferable prognosis, up to 30 per cent of human and canine individuals suffer from intractable pharmacoresistant epilepsy[3]. Factors in people commonly associated with intractability include onset of seizures in the first year of life, number of seizures before initiation of therapy, a high frequency of seizures, tumours and structural brain abnormalities[3].

Possible theories of intractability include the transporter hypothesis, the target hypothesis and AEDs missing the targets[13]. The transporter hypothesis suggests overexpression of AED efflux transporters, such as P-glycoprotein, at the epileptic region, results in ineffective transport of drugs to targets.

The target hypothesis proposes alteration in the cellular targets of AEDs results in reduced sensitivity to treatment. Such targets may include polymorphism-induced alteration of neuronal sodium channels or altered expressions of ?-aminobutyric acid type A ($GABA_A$) receptors

Other hypotheses postulate AEDs are not actually targeting the appropriate processes and mechanisms that may induce epileptic seizures, such as mitochondrial oxidative stress[13]. Although different hypotheses exist, it is likely the causes of intractability are highly variable as well as multifactorial.

Drug-resistant epilepsy has been postulated to be associated with increased risk of premature and unexpected death, injuries, neurobehavioural dysfunction and reduced quality of life[13], [15], [16], [17].

As aforementioned, current treatment options for epilepsy are primarily directed at preventing seizures; that is, the symptom, rather than preventing epileptogenesis.

To develop other potential therapeutic options or an anti-epileptogenic solution, it is important to increase our understanding of the pathophysiological manifestations of epilepsy and seizures. Furthermore, with limited knowledge about the anticonvulsant mechanisms of AEDs, as well at the

mechanisms associated with intractable epilepsy, it becomes increasingly difficult to develop newer drugs with higher efficacy.

Research endeavours in the past, focusing mainly on the genomic, transcriptomic and proteomic nature of epilepsy, has been of limited success. Utilising other research techniques, such as metabolomics, or incorporating several techniques, a systems biology perspective may be the next step to understanding complicated disease models where pathophysiological manifestations are likely to be variable and multifactorial.

Novel discipline

Metabolomics is a novel scientific discipline defined as “the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification”[18].

Profiling of metabolites was first published in literature in the 1950s, but subsequent progress was slow. Rapid improvements in the analytical techniques and data handling systems have significantly increased the potential and applicability of metabolomics in research[19]. Analytical methods, such as nuclear magnetic resonance (NMR) and liquid chromatography/ mass spectrometry, are used to acquire information-rich metabolic data, which allows us to identify various small molecular metabolites present in the metabolome.

Statistical and multivariant data-processing techniques allow for further information extraction and data interpretation[20]. A typical protocol used in a metabolomics approach to interrogate the underlying metabolic differences in various disease models is shown in Figure 1. The metabolome consists of all small molecular weight compounds found in a biological system at any given time. These compounds may include metabolites, such as lipids, small peptides, vitamins, nutrients, hormones, neurotransmitters and other metabolic substrates and products[17], [19].

NMR spectroscopy works on the basis that certain atoms possess an aspect of magnetic spin. When placed in a constant magnetic field the total net spin is aligned with respect to the external magnetic field. Application of a radiofrequency, also known as excitation, causes the net spin to flip to a higher energy state where the orientation of net spin is now opposing the external magnetic field. After absorbing this radiofrequency the net spin spirals back, also known as relaxation, to the original equilibrium state.

During this process, a detectable NMR signal is produced, which is then converted into readable NMR spectra[18], [19], [21]. Molecules are represented by a specific set of lines in the NMR spectra where the position, or chemical shift, is governed by the chemical environment experienced by the nuclei being observed.

Each NMR spectrum represents a unique metabolic profile that contains information of all the

detectable metabolites present at the particular time when the samples were taken.

An example representative 600MHz proton NMR spectrum is shown in Figure 2 containing some of the typical metabolites found in canine urine.

NMR spectroscopy is a comprehensive and non-biased analytical technique that also requires little sample preparation and is inherently quantitative and non-destructive to he samples analysed [18], [22]. However, it also has its disadvantages in comparison to other techniques, such as mass spectrometry (MS) based metabolomics. NMR spectroscopy is far less sensitive (detection limit of one microm to five microm) and requires a large sample size (500ml). Although stronger magnets with higher field strength are able to improve sensitivity, measuring low abundance metabolites can still be problematic[22].

MS analysis, unlike NMR, measures the mass to charge ratio of charged particles. The sample is ionised to produce positive ions and accelerated towards a magnetic field where the ions are then deflected according to their mass and converted into spectral information at the detector. Similar to NMR, MS analysis produces mass spectral data that contains information on the metabolites present. MS analysis offers greater sensitivity, detecting metabolites in the femtomolar to attomolar ranges, and greater selectivity in comparison to NMR-based techniques[23]. MS analysis requires extensive sample preparation procedures, which increases risk of metabolite loss as well as contamination. Variable introduction and ionisation efficiency also means specific metabolite classes may be discriminated and analysis does not provide absolute quantification[19]. However, quantification can be accurately determined by using stable isotope internal standards[22].

Liquid chromatography coupled MS has become a popular experimental setup. The addition of a chromatographic step improves metabolite identification by separating complex mixtures of metabolites.

NMR spectroscopy and MS are both very sophisticated and powerful techniques that are able to generate huge amounts of complex multivariate datasets per study. Different analytical techniques are used, such as chemometrics, to dissect the information and convert it into interpretable spectra in an efficient and non-biased manner. Chemometrics involves data reduction and pattern recognition techniques that are routinely applied in metabolomics studies[19], [22]. Pattern recognition methods can be split into two groups: unsupervised and supervised.

Unsupervised methods, including principal components analysis, involve separating samples based on their metabolic properties and patterns without prior knowledge of sample class.

Supervised methods, such as partial least squares data analysis, apply additional information about sample class, such as clinical data, to compare different predefined groups[22]. In metabolomics, improvements in the use of statistical and pattern recognition methods have meant interpretation of metabolites can be done quickly, easily and inexpensively[19].

While still in its infancy, metabolomics is already beginning to make a significant impact in the field of biology and medicine and is likely to provide new insights and potential in veterinary research.

Although metabolomics is a technique used to analyse metabolites in a sample, there are different approaches in the application to research in different systems. Some of these approaches include the target analysis, metabolite profiling and metabolic fingerprinting [20]. The target analysis involves looking specifically at a small subset of known metabolites using the most appropriate analytical platform for the metabolites of interest. This allows for a more focused investigation on specific metabolic pathways that may provide greater insights into the mechanisms of certain metabolic disturbances[21].

Metabolite profiling, on the other hand, aims to analyse a larger group of metabolites, both identified and unknown with respect to their chemical nature. Finally, the metabolic fingerprinting approach aims to compare the comprehensive metabolic signatures or profiles generated between samples. Metabolites that are able to significantly discriminate between samples are identified and their biological relevance to the system of interest can be elucidated[20].

In a clinical setting, metabolic fingerprinting can be used to compare cohorts of canines with idiopathic epilepsy and compare their metabolic profiles with a normal cohort. Are there any differences? If so what are the biological significances of these differences? Metabolites that are identified have the potential to bring new insight in the mechanisms of epileptogenesis or seizures.

Another interesting comparison would be the metabolic profiles of cohorts representing intractable epilepsy and drug-responsive epilepsy. Metabolites that significantly discriminate between drug-responsive epilepsy and drug-resistant epilepsy act as biomarkers for intractability and, when considering the biological relevance, act as a beacon indicating areas of pathological differences. Similar studies have been reported in disease states including motor neurone disease, Alzheimer's, pre-eclampsia, coronary artery disease, ovarian cancer, breast cancer and hepatic cancer[20].

Summary

Metabolomics is an emerging science with capabilities to discover metabolic signatures of disease and drug response. It takes into account both genetic and environmental contributors to the clinical phenotype (for example, drug treatment, age, lifestyle, gender, chemical exposures, occupational exposures, menstrual and diurnal rhythms, seasonal effects, diet, intestinal bacteria, parasites and even viruses[24].

Also, due to the nature of the analytical platforms involved, many different easily available samples can be analysed, including urine, faeces, plasma, serum, saliva, tissue homogenates and culture medium[16], [24]. The technique is applicable in all disciplines of biological medical and veterinary research and has already been adopted by a number of pharmaceutical companies with drug

development initiatives[19].

Increasing knowledge in the field of metabolomics has meant an increase in improved databases containing useful metabolic data that can be used for future references and comparisons.

Metabolomics presents itself as an extremely useful and powerful scientific research technique. Research at the RVC includes optimising and using a metabolomics approach to understanding canine epilepsy.

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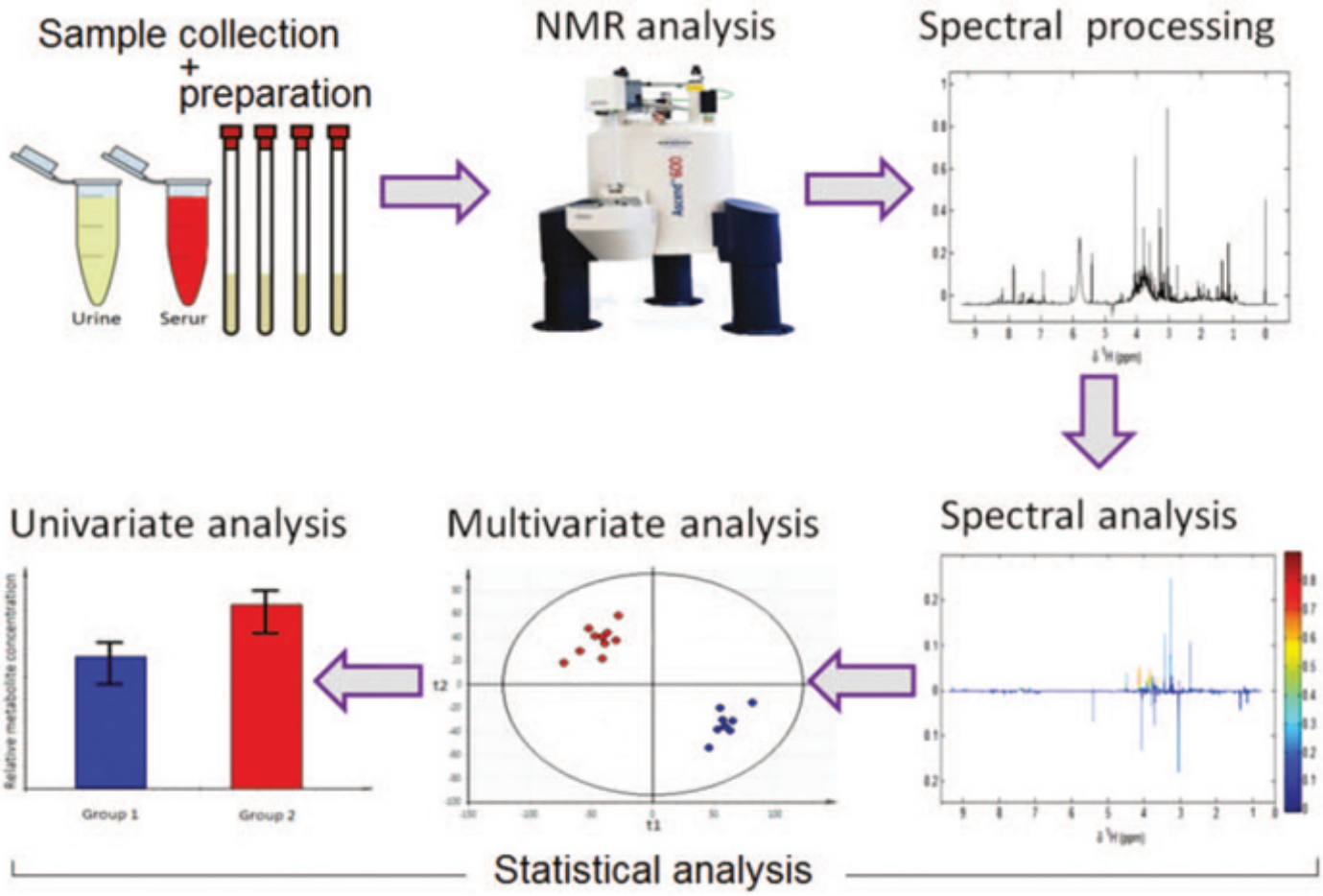

Figure 1. Sample is collected and prepared by removing particulates and adding the internal reference trimethylsilyl propanoic acid (TSP). Samples are then analysed using nuclear magnetic resonance (NMR) spectroscopy. The resulting data are converted into a spectrum where we can apply further spectral processing and analysis. We can also use multivariate statistical data analysis to look at the underlying differences between sample groups. Upon successful detection of specific metabolite changes we can apply further univariate analytical techniques to see specific changes in metabolites. Statistical analysis is applied throughout the data analysis process.

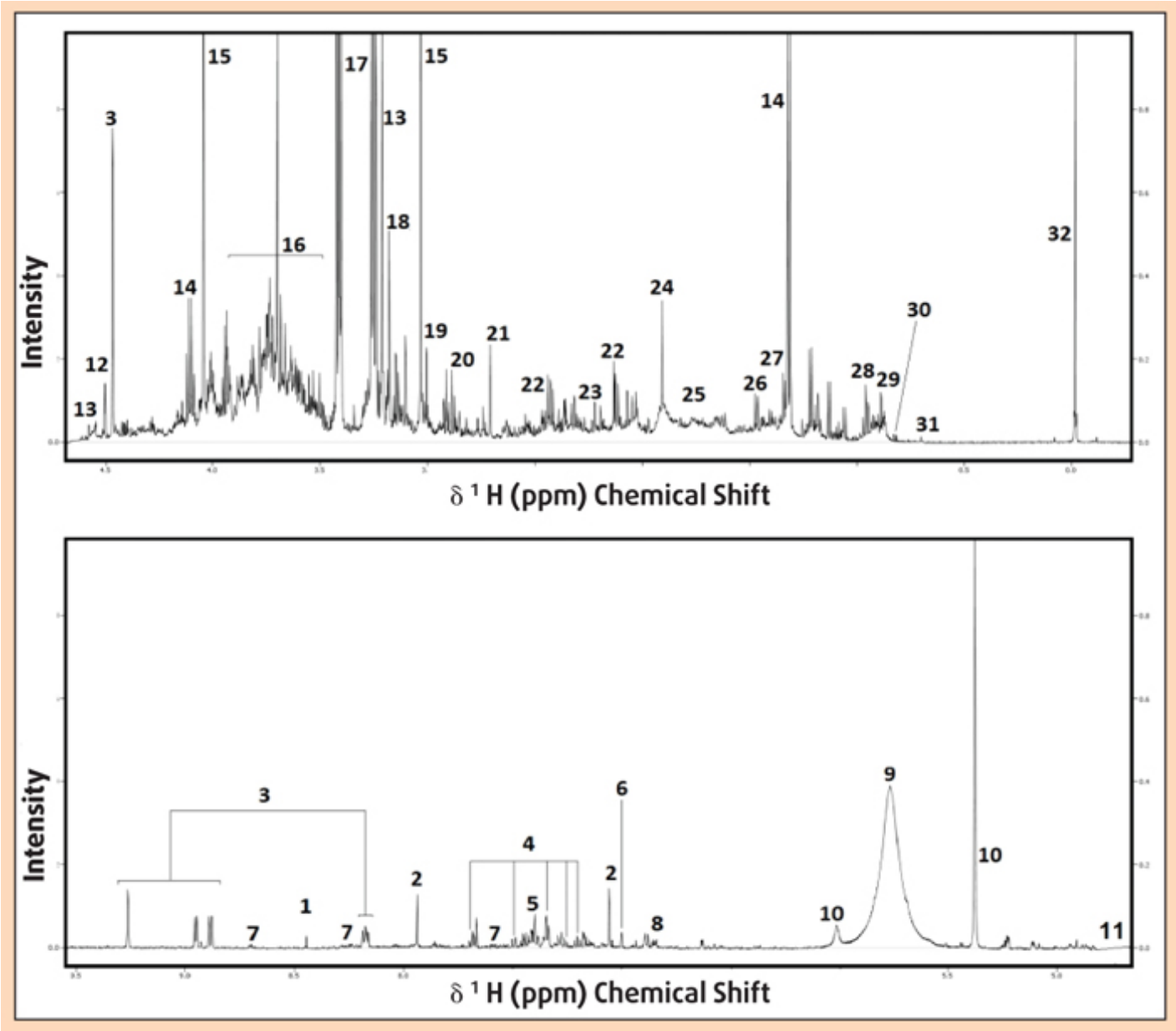

Figure 2. Representative full resolution 600MHz Carr-Purcell-Meiboom-Gill nuclear magnetic resonance spectra of canine urine sample. Metabolites shown include: 1: formate, 2: histamine, 3: 1-Methylnicotinamide, 4: 3-Indoxylsulfate, 5: N-Phenylacetylglycine, 6: Methylhistidine, 7:

nicotinamide, 8: 4-Hydroxyphenylacetate, 9: urea, 10: allantoin, 11: water region, 12: ascorbate, 13: carnitine, 14: lactate, 15: creatinine, 16: sugars, 17: taurine, 18: choline, 19: creatine, 20: trimethylamine, 21: dimethylamine, 22: glutamine, 23: acetone, 24: acetate, 25: arginine, 26: alanine, 27: 2-Hydroxyisobutyrate, 28: 2 aminobutyrate, 29: 2 hydroxybutyrate, 30: hydroxyisovalerate, 31: cholate, 32: trimethylsilyl propanoic acid chemical shift indicator.