The future of Biomarkers and Personalised Medicine in Companion Animal Practice

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S U M M A R Y

The ability to utilise a patient's own genetic expression information to detect the onset of disease, monitor its progression and even suggest possible treatment modalities which have the highest probability of success would undoubtedly provide a mechanism in which to enhance the quality of care and treatment for companion animals. As with all clinical situations, veterinarian decision making is made on a case by case scenario and is based upon an individual's medical history and current disease diagnostic indicators. Having tools available that could help increase the rationalisation process for choosing a particular course of action at the individual level would not only aid the clinical management of these patients but result in enhanced patient care. Tailored or "personalised medicine" as it is referred to, is receiving considerable interest from the human clinical field. New technologies are becoming increasingly available to the medical arena having high-throughput capabilities to perform rapid bio-profiling of individuals. These molecular fingerprints, or biomarkers as they are more commonly known, provide an extremely powerful mechanism in which to exploit diagnostic and prognostic information regarding disease course and therapeutic outcomes for individual patients. The potential of these technologies to detect individualised molecular fingerprints are not restricted to only human medicine. Biomarker technologies are now being translated into the veterinary arena and offer the same potential to the veterinary practitioner as they do to their counterparts within human clinical medicine – that is the ability to personalise the treatment of individual patients resulting in enhanced patient care to unprecedented levels.

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INTRODUCTION

Phenotypic signature patterns - biomarkers

The genome represents the total genetic composition of a biological organism and through co-ordinated temporal (time) and spatial (location) gene expression the development of cells and tissues with specialised function occurs. The overall result is the production of an organism with defined attributes and accounts for both species differences and individual breed characteristics. Enabling subsets of genes to be specifically activated and de-activated is one of the primary mechanisms in which an animal may control its normal physiological function. The products of gene expression (RNA, protein, carbohydrate, lipid etc) can be considered as an organism's phenotype and the ability to monitor these signature patterns during normal physiological processes would provide a useful mechanism in which to monitor overall functional status [1]. Deviation in the expression of these molecular fingerprints could therefore represent the first sign in the development of a pathological state. The production of diagnostic tools which have the ability to profile

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a patient's molecular fingerprint to monitor pathological changes would provide a novel mechanism in which to monitor disease onset and its progression [2].

Biomarkers can be described as phenotypic signature patterns of a given physiological state(s). As such, they have the potential to represent a variety of biological processes which may include, for example, normal or diseased conditions. In principle these molecular fingerprints can be elicited from any biomolecule which is expressed from an organism's genome (e.g. RNA, protein) and by extrapolation therefore, exist within any tissue type implicated to a particular physiological process. Biomarkers are receiving considerable attention by clinical practitioners of human medicine due to their translational application as diagnostic or prognostic indicators [3]. They have been used as phenotypic indicators for early disease detection and progression in a wide range of pathologies extending over several medical disciplines e.g. oncology, renal, cardiac, immunological, neurodegeneration etc [4].

New technologies have arisen to meet this growing demand for rapid biomarker identification. It is envisaged that they will be able to provide greater levels of sensitivity and specificity than is currently afforded by conventional disease markers [5]. An ability to identify biomarker patterns from specific patients with defined pathologies could therefore, offer the promise of a personalised approach to healthcare [6]. There would be many advantages of adopting such a strategy to clinical practice. It could for example facilitate the selection of treatment regimes in which a patient may possess a high probability of responding whilst avoiding therapeutics that are likely to be ineffectual and/or likely to show significant toxicity to non-target tissues [7]. Providing such tools to clinical practitioners would provide a mechanism in which to rationalise the clinical decision making process for a given patient using data derived from diagnostic biomarker patterns.

Owners of companion animals and the veterinarians who treat them are now demanding ever increasing standards of quality care administered to pets. Translating biomarker technologies with diagnostic/predictive capability to the veterinary field would provide one such mechanism in which to facilitate this process. The rationale for such a directed approach is simple. Each patient which is presented clinically to a veterinarian will be assessed at an individual level, i.e. on a case by case scenario. Factors such as previous medical history, current clinical manifestations, type of animal, and risk to particular pathologies will then set in motion a series of diagnostic investigations. Integration of all of these factors would facilitate the initiation of a course of clinical action with concomitant patient monitoring for suitable therapeutic response. Such a dynamic situation represents a highly challenging environment for clinical practitioners, especially veterinarians who unlike their clinical counterparts within human medicine, have to deal with a variety of animal species with their predisposition for particular diseases. Biomarkers may offer the potential therefore to identify key disease indicators at the individualised level for a particular species of animal.

Single versus multiple biomarkers

Phenotypic differences within an individual companion animal are not restricted to species, age, sex, breed, single nucleotide polymorphisms (SNPs) but also within an individual's metabolic state, disease and immune status etc [8]. While certain expression patterns within an organism are relatively constrained (e.g. proteins associated with the differentiation status of a specialised cell such as a cardiomyocyte) the temporal and spatial pattern of other genes need to be co-ordinately activated, repressed and their gene products modified and turned over in response to meet the daily physiological changes required to maintain homeostasis. Environmental factors can also influence phenotypic expression patterns throughout the life of an animal [9]. A patient therefore represents a mixture of conserved and dynamic subsets of gene expression patterns. How is it possible with such biological heterogeneity therefore to identify biomarkers that will be of clinical relevance? The answer is believed to reside in the use of multiple rather than single biomarkers [10]. Multiple biomarkers represent a composite pattern populated by several key differentiators which indicate the normal versus the pathological state. This approach is expected to achieve the levels of diagnostic redundancy needed for personalised veterinary care. Unless a marker is specifically associated with a diseased state and is not expressed in any other tissue type, it is unlikely that clear demarcation between normal and diseased states will ensue using a simple presence/absence diagnostic tool. Diagnostic assays that can accurately distinguish between the number of true positive cases for a disease indication (sensitivity) and true negative cases (specificity) will be essential if the field of veterinary medicine is to move towards personalised healthcare. Given the nature of genetic variation not only from species to species but from breed to breed, it is likely that multiple biomarkers will be essential to deal with inter and intra group variation.

Biomarker identification – the post genome technologies

In 2004 the International Human Genome Sequencing Consortium published a highly accurate sequence annotation of the human genome from approximately 3 billion base pairs of genetic code with 99% sequence coverage [11]. It is believed that the human genome encodes for some 20,000-25,000 genes and taking into consideration such events as alternative splice variants and post-translational modifications there may be somewhere in the order of 500,000 to 1 million gene products in total. In 2003 an initial publication regarding the genomic sequencing of canine was reported [12]. With approximately 2.4 billion base pairs and an estimated 20,000 genes, it is likely that the total number of gene products will be similar to that found in humans. Identifying biomarkers with clinical relevance to the veterinary field will be extremely challenging given not only the number of biological products with biomarker potential for a given animal species with, but also the different separation and isolation characteristics which will be associated with different classes of biomolecule (e.g. RNA, protein, lipid). In order to make the problem tractable, several areas have emerged to analyse each subclass of major molecule in this post-genomic area. For example the study of RNA transcript expression is referred to as transcriptomics [13], while the analysis of

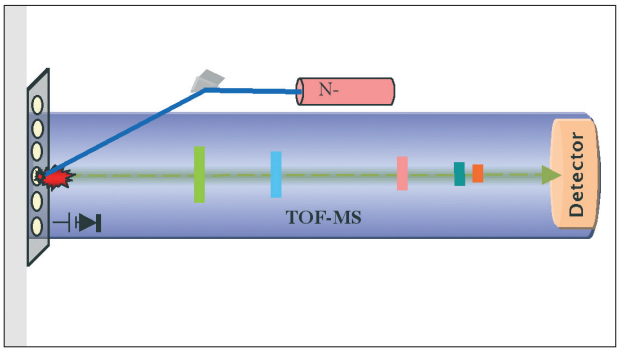


Figure 1: A Time-of-Flight (ToF) mass spectrometer. A laser is fired at the target support containing the clinical sample and the proteins are ablated from the surface (red spot). The molecules are then accelerated in a flight chamber and hit the detector at rates which are inversely proportional to their mass.

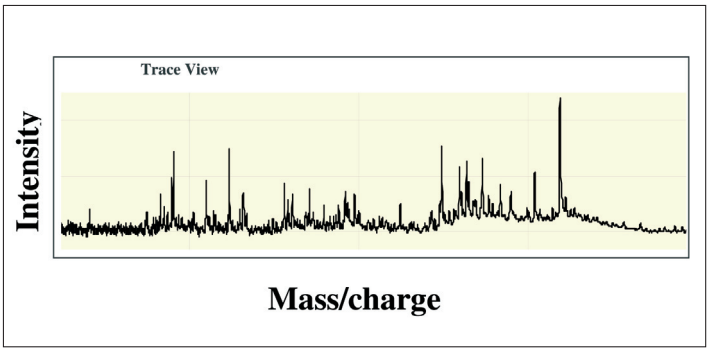


Figure 2a: Proteomic profiling derived via the mass spectrometric analysis of proteins from a biological sample. The y-axis represents relative intensity value while the x-axis denotes the mass:charge value for each peak.

metabolite expression is known as metabolomics [14]. The study of protein expression patterns whether in single cells or complex tissues such as blood plasma, is known as the field of proteomics [15, 16, 17]. While several technologies have been developed to deal with the complex biological diversity of protein populations (e.g. antibody/antigen microarrays; surface plasmon resonance), one approach using mass spectrometry is proving to be an extremely powerful tool in the search for diagnostic biomarkers with clinical relevance [18, 19, 20, 21].

Seldi

Mass spectrometry is an analytical tool which has the ability to accurately measure the mass of a molecule and the time-of-flight instruments (ToF) have been proving to be extremely useful in the search for disease related biomarkers (Figure 1) [22, 23, 24, 25]. Proteins entering the mass spectrometer are ionised and accelerated down a field free flight chamber where they hit a detector. The speed at which molecules "fly" is inversely proportional to their mass and as such small molecules arrive at the detector faster than larger molecules. In this manner, a relative intensity spectrum can be produced from a clinical specimen in which intensity is represented on the y-axis and m/z (mass:charge) is denoted on the x-axis (Figure 2a). A simple comparison of two mass spectra e.g. one derived from a serum sample taken from a dog without cancer and one taken from an animal with cancer may enable differences in protein expression patterns to be rapidly identified (Figure 2b). These proteomic based biomarkers may therefore form the basis of a diagnostic test. Different methodologies can be adopted to introduce biological specimens into mass spectrometers for proteomic analysis and one key approach which has received considerable interest is where the biological sample is prepared as a mixture with an energy absorbing molecule called matrix. The biological sample and matrix are co-crystallised on a solid support surface (the process is referred to as MALDI (Matrix Assisted Laser Desorption/Ionisation)) and the mixture analysed by ToF mass spectrometry. The advantage of such an approach

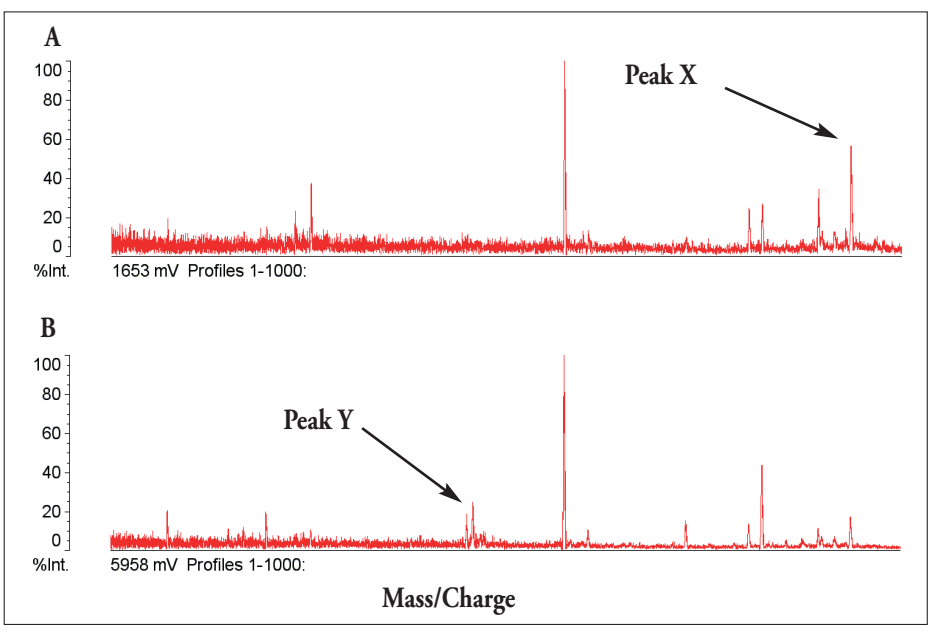


Figure 2b: A serum biomarker profile taken from a dog with no pathology (A) and a dog with confirmed cancer (B). In Figure 2A peak X is noted to have a higher relative intensity value than the animal with cancer. Figure 2B shows the presence of peak Y which is absent from the animal without disease.

clinically is that high throughput automated procedures can facilitate the screening of hundreds to thousands of patient samples in a relatively short time.

In MALDI based applications the biological sample is prepared for mass spectrometric analysis prior to deposition on the MALDI target plate through a series of clean-up processes enabling the complexity of the clinical specimen to be reduced and mass spectrometric analysis greatly simplified. An alternative approach is to use a solid support which has a chemically modified surface (e.g. possessing antibody coating for the selection of particular epitopes or having biochemical modifications for the preferential selection of particular classes of proteins/peptides (e.g. hydrophobic, hydrophilic, cationic, anionic etc)). The use of this type of modified solid support or "protein chip" is known as SELDI (Surface Enhanced Laser Desorption/Ionisation) and it has a number of advantages over conventional MALDI. These include both rapid sample clean-up (as it occurs on chip rather than off) and mass spectral reproducibility. As with conventional MALDI, matrix is applied to the protein chip to form a protein crystalline structure and the biological sample is analysed directly using ToF mass spectrometry. The SELDI protein chip technology has been used to characterise biological material from a variety of sources in the search for biomarkers with clinical relevance 26, 27, 28, 29]. Clinical precursor material which has been analysed includes serum, plasma, solid tissue, urine, cerebral spinal fluid etc. The versatility and adaptability of the system makes it highly suitable for clinical applications where the types of biological material being presented for diagnostic analysis are varied and a requirement to process large sample numbers in relatively short time periods is necessitated [30]. As such the potential for developing novel diagnostic tools for veterinary medicine is significant using this type of approach.

Bioinformatics – computer algorithms for biomarker identification

In the search for biomarkers with clinical diagnostic/prognostic capability, bottlenecks were originally confined to the lack of reproducible throughput of hundreds of patient samples.

Squamous cell carcinoma in a cat.

Identification of potential markers necessitates the use of hundreds of specimens in order to develop statistically confident biomarkers which could form the basis of novel assays. Originally this was extremely difficult as many of the proteomic platforms did not lend themselves immediately to high throughput analysis. However, the drive to identify disease related biomarkers has resulted in the emergence of new technologies with high throughput capability and integrated workflow processes. The result was no longer a constraint on sample processing and data generation, but an ability to process the large volumes of proteomic data which are necessary for the identification of clinical biomarkers. One mechanism to deal with this issue was to implement the use of computer programmes which have an ability to recognise expression patterns associated with particular phenotypes e.g. normal or disease conditions [31, 32]. To date a variety of approaches have been implemented in order to "mine" data for biomarker expression patterns. Data mining, as it is referred to, assists in rapidly identifying the most important protein candidates having diagnostic /prognostic potential. Methodologies such as CART (Classification and Regression Tree analysis), PCA (principal components analysis), and artificial neural networks have been used to assist in biomarker identification [33, 34]. The power of computational analysis for clinical biomarkers lies in the fact that they can analyse literally millions of data points in a relatively short time providing a cohort of proteins which may be useful as predictive indicators. Once key biomarkers have been identified, these algorithms have been utilised to predict the pathological status of additional blind clinical samples with high degrees of accuracy.

CLINICAL APPLICATIONS OF SELDI PROTEIN CHIP TECHNOLOGY TOWARDS BIOMARKER IDENTIFICATION

Ovarian cancer represents a highly aggressive gynaecological tumour which is normally innocuous to the patient until presentation at late stages. Clinical manifestation is likely to coincide with metastasis and increased biological aggressiveness, making surgical resection and therapeutic intervention extremely difficult. Early detection of ovarian cancer results in high rates of patient cure. The need for diagnostic biomarker assays with greater sensitivity for detecting true positive cases is therefore justified. In 2002 a seminal paper published in the Lancet by Petricoin et al [35] presented data to show how proteomic profiling using SELDI protein chip technology followed by analysis by ToF mass spectrometry could be utilised to produce a "protein fingerprint" of serum. Initial molecular profiling was performed upon 50 patients with ovarian cancer and 50 control individuals who where either disease free or had benign conditions. Using a "supervised" learning algorithm (a computer program in which the user informs the algorithm which profiles are derived from cancer patients and which are not; in this manner the programme has a basis upon which to identify biomarker peaks associated with each diagnostic class "cancer"

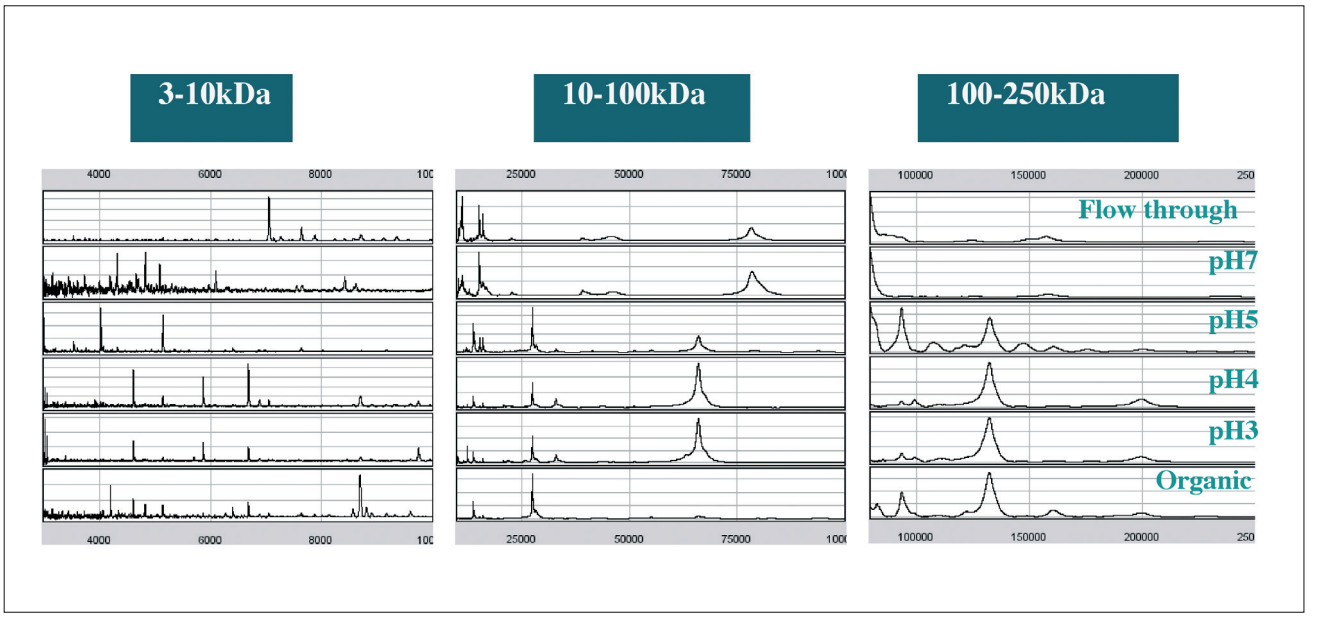


Figure 3: A schematic of how different peaks can be detected using mass spectrometric analysis on SELDI chips using anion exchange chromatography in order to fractionate. The mass:charge value is presented on the x-axis while relative intensity and elution phase are shown on the y-axis.

and "non-cancer") to train an iterative searching program, 116 blind serum sample proteomic profiles (50 cancer and 66 non-cancer) were then presented to the trained architecture. From this study 100% of ovarian cancer patients and 95% of non-cancer individuals were correctly classified. While flaws were identified in this original study [36] it proved that serum biomarkers could be used as basis for classifying patients diagnostically. Since then, biomarker profiling of serum has been used to discriminate patients with cancer from those without in a number of oncological indications including prostate [37], breast [38], ovarian [5] and lung [39].

Mian et al have used a similar profiling methodology in conjunction with supervised training algorithms to classify serum samples taken from melanoma patients with either stage I or stage IV disease [40]. Two hundred and five serum samples from 101 early-stage (American Joint Committee on Cancer [AJCC] stage I) and 104 advanced stage (AJCC stage IV) melanoma patients were profiled using SELDI protein chip technology and linear ToF mass spectrometry. 109 samples (representing approximately equal distribution of both stages) were used as a training set for an artificial neural network (ANN) computer algorithm. ANNs mimic animal brains in that they learn through an iterative process of trial and error. Once training was completed the algorithm was then used to classify 96 blind proteomic profiles derived from the remaining group of stage I and stage IV patients. It was found that 88% of samples were correctly classified. Extending the study further, these authors profiled the serum taken from 55 patients with stage III disease and one year clinical follow-up. 28 patients were known to have progressed to stage IV disease whereas 27 did not. Using biomarker analysis, patterns of expression could be identified by the ANN enabling 80% of the patients to be correctly classified as patients who either do or do not progress to stage IV. The potential of identifying at risk individuals for disease progression using biomarkers was shown to be feasible using this technological approach.

Biomarker identification using SELDI protein chip technology for disease identification has not been restricted to the use of serum. It has been applied to tissue sections e.g. for grading tumours [41] or identification of biomarkers associated with disease, cell lines for chemo-response [42], urine for biomarkers associated with bladder cancer [43] and cerebral spinal fluid. The potential for human personalised medicine using these types of novel approaches to assess risk of disease onset, its progression and even therapeutic response for individuals is promising. By analogy their implementation into veterinary practice has the potential to aid clinical decision making processes for patients to an equal degree.

Application of SELDI protein chip to canine cancer serum fingerprinting

In order to ascertain the potential of SELDI proteomic profiling for biomarker discovery in veterinary clinical samples, these authors conducted a proof of principle study using serum taken from dogs with a variety of cancers (mast cell/lymphoma) versus dogs without disease. Each serum sample was fractionated initially using anionic exchange chromatography (enriching for proteins which possess negative regions) and eluted with sequential buffers of increasing pH (pH3, pH4, pH7, pH9 and organic). Fractionation of any complex biological sample (tissue/serum etc) is critical if clinically important biomarkers of low concentration are to be detected. It has been widely reported that high abundant molecules e.g. serum albumin, IgG may

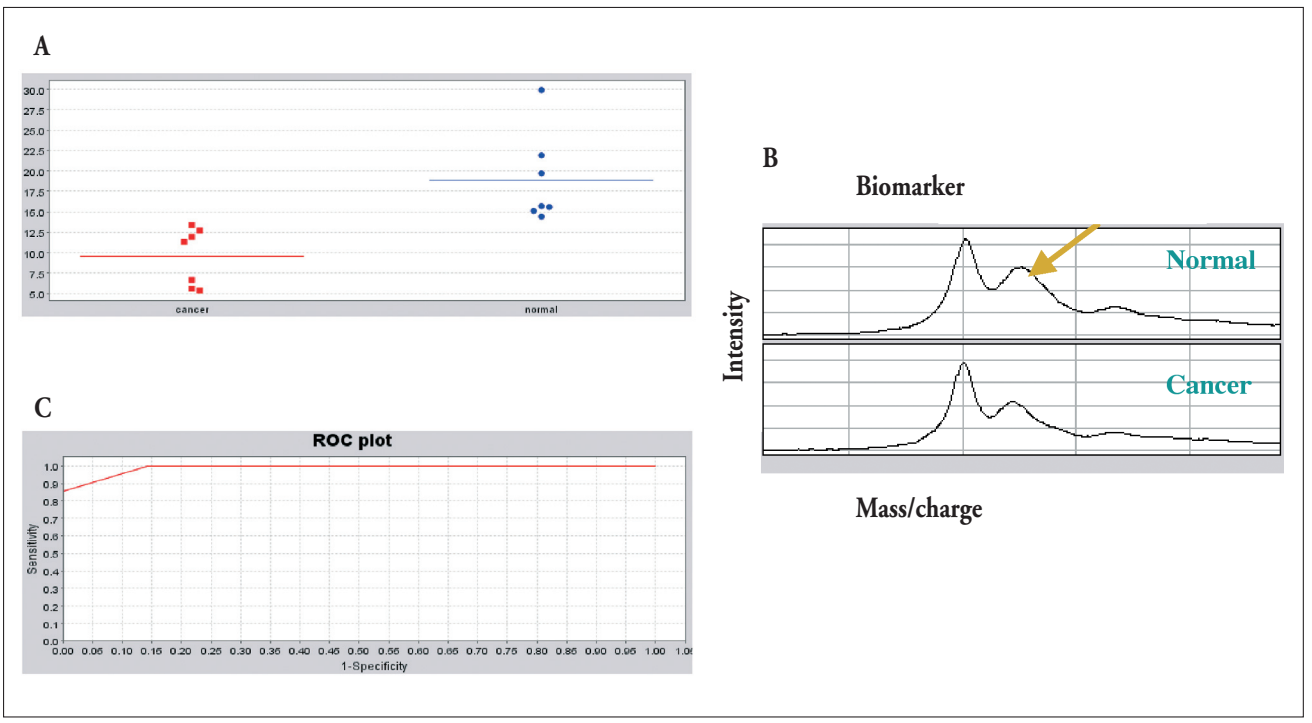
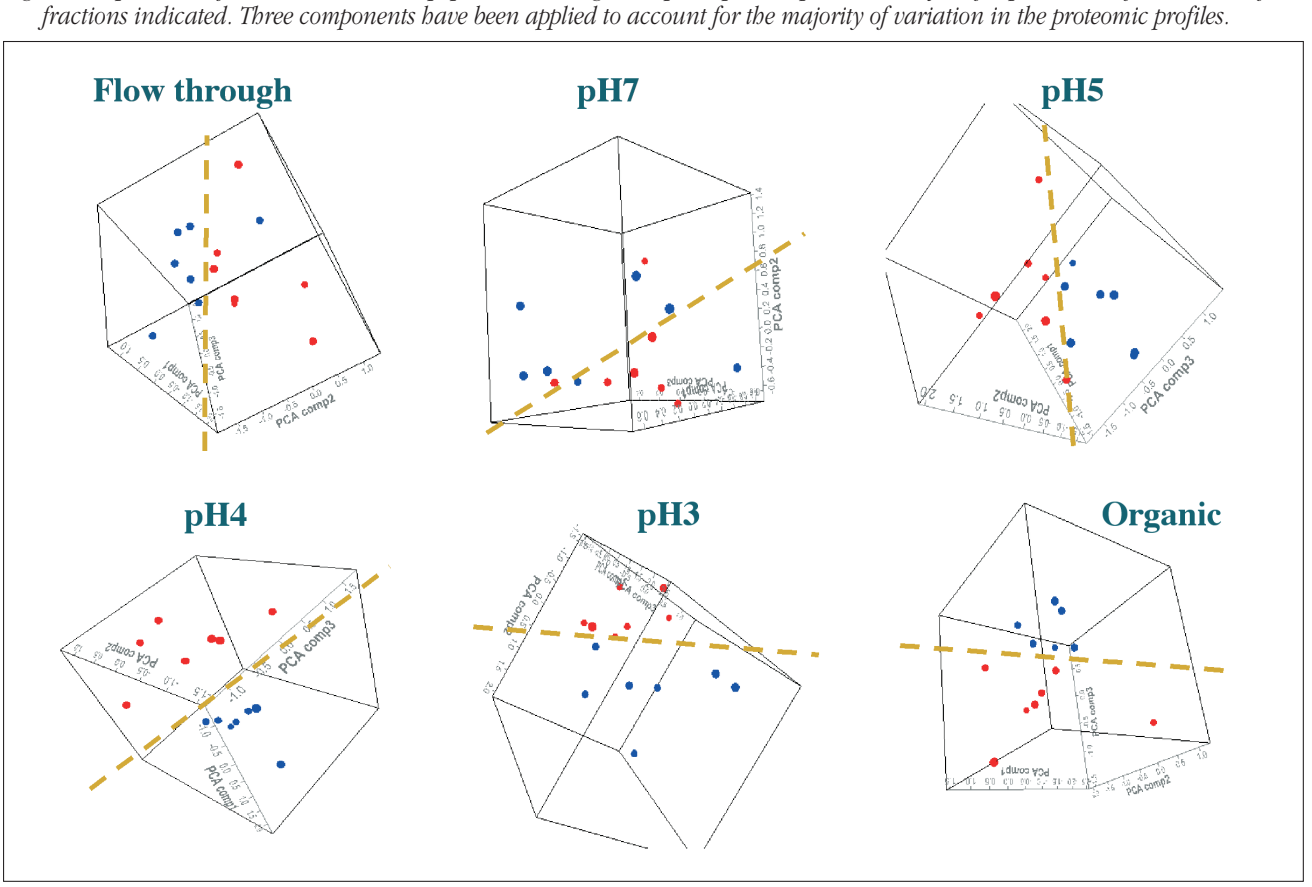


Figure 4a: Mean intensity values for a highly significant serum biomarker are presented for the cancer and normal populations.
Figure 4b: Visualisation of the reduction in relative intensity value for the biomarker between normal and cancerous serum.
Figure 4c: A receiver operating characteristic (ROC) plot showing the ability to detect true positives (sensitivity) from false positives (1- specificity).

Figure 5: Separation of cancer and normal populations using PCA (principal components analysis) for peaks identified in each of the fractions indicated. Three components have been applied to account for the majority of variation in the proteomic profiles.

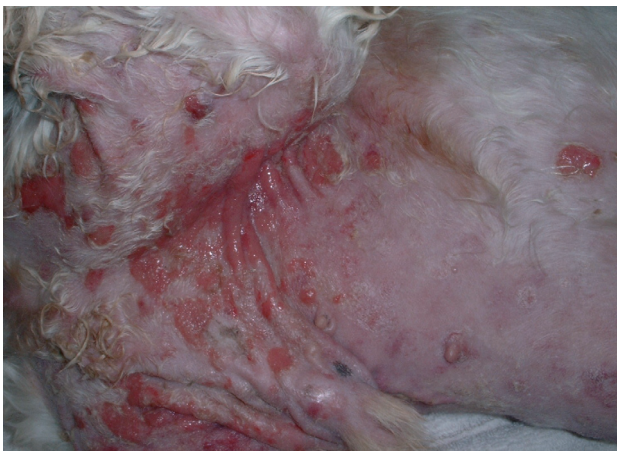


mask the detection of disease associated biomarker proteins. The removal of these molecules by reducing sample complexity may therefore facilitate the detection of novel disease related biomarkers which are present in very low amounts. Figure 3 shows how a serum sample can be fractionated into alternative protein components using only a single type of SELDI chromatography (anionic exchange). A comparative analysis between fractionated and un-fractionated serum indicated that a greater number of peaks could be detected in the fractionated serum compared to the non-fractionated sample (321 versus 134 peaks respectively – data not shown). The greater the number of protein peaks detected the higher the probability that one or more may provide the basis of a diagnostic biomarker pattern with disease association.

In order to identify peaks having potential utility as biomarkers for cancer, statistical analysis using t-testing was performed to select candidates which had significant differences ($P<0.05$ level) between normal and cancer patient serum samples. Initial data passing revealed that 81 peaks had statistically significant p-values at the $P<0.05$ level (data not shown). An example of one of the significant peaks is presented in Figure 4. A scatter plot of intensity values for the biomarker in both normal and cancer associated serum samples is provided in Figure 4a. This peak is significantly down-regulated in cancer serum samples ($P<0.001$) compared to non-diseased animals. Figure 4b shows the biomarker which has been identified via automated peak detection software. Figure 4c presents data in the form of a ROC (Receiver Operating Characteristic) curve which produces a measure of the ability of a biomarker to discriminate between true positives i.e. cancer patients (also referred to as sensitivity) and false positive i.e. negative patients which are classified as positive (this is calculated as 1-specificity). The AUC (Area Under the Curve) value was calculated to be 0.99 indicating good demarcation of this biomarker peak to identify true positives from false positives within the cohort of samples tested. As biological variation increases e.g. breed, sex, tumour type then it is likely that single markers which show extremely good promise within the early stages of diagnostic evaluation do not have sufficient power to deal with the complexity seen clinically.

Additional data analysis using principal components analysis (PCA) (a statistical approach to identify principal components which can account for as much of the variation in data between the two populations) is presented for each of the fractions (Figure 5). The data would suggest that all fractions can be separated extremely well (with the exception of pH 7) into cancer and normal populations using this type of approach using candidate biomarker ions which were initially identified as having statistically significant p-values.

The data presented in this initial analysis has indicated that serum biomarkers can be rapidly identified which are capable of distinguishing animal patients which either do or do not have cancer. The ability to develop novel biomarker assays for companion animal diseases such as cancer is likely to afford the same clinical benefits to the veterinary field as will be seen in the human field. Biomarkers do not simply represent a paradigm in which to manage patients clinically. The benefits of biomarker identification are far reaching and include the possibility of choosing the most appropriate form of therapy for a given individual, developing novel therapeutics and ultimately understanding the cause of disease onset and progression.



Epitheliotrophic cutaneous T-cell lymphoma (mycosis fungoides) in a dog.

CONCLUSIONS

Human clinical medicine is moving towards the exploitation of genetic information via post-genome technologies. Biomarker assays represent one key evolutionary product from this era. Veterinary medicine is also capitalising upon these advances as the profession moves rapidly to exploit post-genome information and facilitate its translation into a clinical setting. The results for companion animal practice both in the short and medium/long-term will be the production of significantly improved diagnostic assays for a wide range of clinical pathologies. Additionally, it should facilitate production of novel therapeutics with greater specificity and less toxicity. This will ultimately translate to a more directed approach to patient care than can be afforded with current veterinary medical practice and aid veterinarians in their decision making processes.

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