Proteomic Approaches for Diagnostics of Canine and Feline Dementia

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Cognitive dysfunction syndrome (CDS) in pet animals constitutes a pressing problem of the modern society. Millions of senior dogs and cats undergo age-related behavioral changes that impact their social interactions. Currently, it is difficult to discriminate between normal aging and dementing processes. The situation is akin to that in humans; however, human medicine receives enormous resources that resulted in a set of current diagnostic criteria including a number of dementia assessing scales, diagnostic assays, and novel potential biomarkers. While animal well-being is not in the limelight of societal interest, the dementia diagnostics starts to catch up (Madari et al. 2015; Schütt et al. 2015). Nevertheless, biochemical markers related to the animal dementia are underdeveloped, despite the fact that dogs and cats provide natural models for human dementia (Bosch et al. 2012; Chambers et al. 2015).

7.1 Tissue Types for Biomarker Identification and Quantification

Biochemical diagnostics usually focuses on the body fluids as the most accessible sources of biological markers of disease. The concentrations of potential brain-derived biomarkers in the body fluids substantially decrease in the following order: (brain)–cerebrospinal fluid–blood–urine. Saliva and tears complete the list, being positioned somewhere between blood and urine; nonetheless, their diagnostic utilization is not much exploited.

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G. Landsberg et al. (eds.), *Canine and Feline Dementia*,
DOI 10.1007/978-3-319-53219-6_7

7.1.1 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) protects the central nervous system (CNS) from physical stress and plays an essential role in homeostasis of the brain and regulation of neuronal functioning. CSF is a clear liquid of extremely low cellularity (0–8 cells/μL). It is secreted by cells of the choroid plexus and ependymal cells that line the ventricles and is absorbed into the venous system in the subarachnoid space (Johanson et al. 2008). Its composition is determined on one hand by the relatively free exchange of proteins, peptides, and metabolites with the brain tissue and on the other by a highly restricted and regulated exchange with blood across the blood-CSF barrier (McComb 1983; Iliff et al. 2012; Tarasoff-Conway et al. 2015).

Since CSF is in direct contact with the brain interstitial fluid (ISF) that soaks the neurons, biochemical changes in the brain are reflected in CSF. Furthermore, CSF has low protease activity, and most proteinaceous molecules do not change upon collection provided, of course, the collected CSF sample is not contaminated by blood. Therefore, CSF should be the best source of biomarkers that reflect the pathological changes of the brain (Blennow et al. 1993).

The total protein concentration reaches around 0.3 mg/mL, which increases with age (Maurer 2010). In dogs and cats, protein concentration >0.8 mg/mL indicates alteration of the blood-brain barrier and/or increased local synthesis. Albumin (~0.2 mg/mL) constitutes around 67% of the total CSF protein; beta-trace, prealbumin, immunoglobulins, and transferrin make up additional 27%. On the other side of the CSF protein, rosters are such neuron-specific proteins like myelin basic protein, S-100, cytokines, and bioactive peptides with concentrations in sub-pg/ml levels. CSF concentrations of dementia-related proteins like amyloid β 1-42 (Aβ42) and tau are around 550 pg/mL (Borghys et al. 2014) and 30 pg/mL (Roerig et al. 2013), respectively.

Thus, the concentrations of proteinaceous molecules in CSF span at least nine orders of magnitude. Eighty percent of CSF proteins, the most abundant ones, are blood derived, and only 20% come from the brain ISF. The current and future dementia markers are expected to be found in the low concentration range of >3000 CSF proteins and peptides, resulting in an enormous difficulties for analytical quantifications (Schutzer et al. 2010; Guldbrandsen et al. 2014).

7.1.2 Blood (Plasma and Serum)

Blood (plasma or serum) is far more accessible than CSF; however, the analytical complexity of plasma (serum) appears even higher than that of CSF. First, the concentration range of the individual proteins spans 10–11 orders of magnitude (Anderson and Anderson 2002). Second, the total plasma protein concentration is ~55–75 mg/mL, 200-fold higher than in CSF, and thus any brain-derived proteins will be highly diluted. For example, Aβ42 is present in the dog plasma at 25–75 pg/mL, tenfold lower than in CSF (Gonzalez-Martinez et al. 2011; Schütt et al. 2015).

Third, proteins and peptides might have a short half-life in plasma due to fast renal clearance. Blood contains relatively high proteolytic and other enzymatic activities causing most intracellular proteins released into the bloodstream to undergo degradation and/or modification by proteases and other enzymes, and, therefore, for most of the biomarker candidates, the half-life in the blood is unknown (Werle and Bernkop-Schnürch 2006).

Nevertheless, major human neurodegenerative diseases have a substantial neuroinflammatory component, and some biomarker signatures seem to be connected to the peripheral immune system (reviewed in Chiam et al. 2015; Zafari et al. 2015). It makes, therefore, sense to exploit plasma (preferred over serum) for identification of novel biomarkers.

7.1.3 Urine, Saliva, and Tears

Some of the brain-derived proteins and peptides might find their way to other body fluids like saliva, tears, or urine. For example, neuronal protein tau associated with neurofibrillary pathology and α-synuclein and DJ-1 proteins connected to Parkinson's disease were detected in saliva samples in humans (Devic et al. 2011; Shi et al. 2011). It remains to be seen, however, whether their amount reflects pathological processes in the brain.

Similarly to the blood, the urine is a fluid relatively easy to collect and thus promising source of biochemical markers. Over 1500 proteins have already been identified in the urine (Adachi et al. 2006; Rodríguez-Suárez et al. 2014), and it is not excluded that some of the more hydrophilic proteins and peptides might get accumulated there due to their fast renal clearance.

7.2 Biomarker Quantification Methods Used in Diagnostics

Biomarkers for human dementia, especially Alzheimer's disease, have been analyzed for almost 20 years now, but none of the tests currently available gives 100% specificity and sensitivity. Nevertheless, some of them have been validated for humans and provide substantial help in a differential diagnosis of neurodegenerative disorders (Dubois et al. 2014; Olsson et al. 2016).

7.2.1 Immunoassays

Immunoassays were historically the first techniques that achieved the sufficient sensitivity and specificity to detect the identified biomarkers in body fluids. The widespread ELISA (enzyme-linked immunosorbent assay) format usually requires two antibodies (sandwich ELISA): one is immobilized on a microtiter plate and serves as a capture antibody to fish out the biomarker from the sample and the other is labeled and serves as a detection antibody for the captured molecule. The signal can be amplified by

using a third antibody or using a biochemical amplification system. The ELISA tests for neuronal protein tau and Aβ42 in CSF were first developed in the 1990s (Vandermeeren et al. 1993; Hulstaert et al. 1999). The CSF-based tests INNOTEST® hTAU, INNOTEST® PHOSPHO-TAU$_{(181P)}$, INNOTEST® β-AMYLOID$_{(1–40)}$, and INNOTEST® β-AMYLOID$_{(1–42)}$ (Fujirebio Europe, Ghent, Belgium) remain the most useful and characterized assays in quantifying the biomarkers of human neurodegenerative disorders and can be used in canine and feline diagnosis of dementia. Other immunoassays include those for microglia and astrocyte marker YKL-40, neurofilament light chain, alpha-synuclein, DJ-1 protein, and several others (reviewed in Blennow et al. 2016; Olsson et al. 2016). These tests are used in experimental diagnostics, identification of disease subtypes and staging, differential diagnostics, and stratification of patients in clinical trials (Lleo et al. 2015).

Due to the high homology between human, dog and cat variants of these proteins, many of the assays can be immediately applied to use in animals. However, suitable population studies determining the normal and disease-associated values need to be performed prior to drawing any meaningful diagnostic conclusions.

7.2.2 Multiplex and High-Sensitivity Immunoassays

With the increasing number of ELISA tests, it soon became clear that the sample consumption and labor were exceeding the practical laboratory throughput. Modifications were sought to accommodate more than one analyte per well (e.g., Aβ Triplex assay, U-Plex Chemokine 25-plex, Canine Proinflammatory kit, all from Meso Scale Discovery, Rockville, USA) or to use color-coded (magnetic) beads with immobilized capture antibodies, e.g., xMAP® technology from Luminex, for up to 500 analytes in one assay (Ellington et al. 2010).

Standard ELISA assays and their multiplex modifications have lower limits of quantification around 10–100 pg/mL. This prevents quantification of neuronal proteins in plasma, since e.g. tau protein and S-100 calcium-binding protein B are present in sub-pg/mL levels (Shahim et al. 2014). Therefore, highly sensitive diagnostic assays are being developed.

The measurement of tau and S-100 calcium-binding protein B in blood (plasma) was allowed thanks to an ultrasensitive, digital, bead-based immunoassay called SIMOA (single-molecule array) developed by Quanterix Corp. (Boston, USA) and published recently (Rissin et al. 2010). The assay is actually a standard sandwich ELISA assembled on magnetic beads, which are then transferred to a plate containing ~300,000 microwells for quantification. The wells accommodate only 1 bead, which allows quantification of each individual molecule of the analyte (hence "digital" ELISA) leading to ~1000-fold, increases in sensitivity over the conventional assays. Aβ42 was measured using SIMOA in sera of patients resuscitated after cardiac arrest, and elevated levels were found to correlate with the clinical outcome (Zetterberg et al. 2011). The SIMOA assay was also used for the quantification of tau in serum of these patients, and the elevated levels could have utility for

hypoxic brain injury assessment and prediction of cerebral function outcome (Randall et al. 2013). Plasma tau was also assessed with the relation to Alzheimer's disease diagnosis and was found to be mildly elevated but not useful as a diagnostic value (Zetterberg et al. 2013). However, the ultrasensitive measurements of tau in the plasma of people suffering from head injuries (concussions, blasts, etc.) or brain hypoxia proved useful for the assessment of the clinical outcome (Ling et al. 2015; Gren et al. 2016).

Another ultrasensitive platform, single-molecule counting (SMC, from Singulex, Alameda, CA, USA), is a bead-based sandwich immunoassay, where individual fluorescently labeled detection antibody molecules are counted with a confocal detection system. The assay has been used for the quantification of neuronal visinin-like protein-1, neurogranin, and Aβ oligomers in the human CSF and showed correlation with the progression of brain atrophy in Alzheimer's disease (Tarawneh et al. 2012, 2015, 2016; Yang et al. 2015).

In a proximity extension assay (PEA, developed by OLINK Proteomics, Uppsala, Sweden), two antibodies carrying partially overlapping complementary oligonucleotides are used for one biomarker. These antibodies are mixed with the sample; by binding the same molecule they get to close proximity, the oligonucleotides bind each other and allow detection of the signal by quantitative PCR. This setup permits quantification of multiple different analytes in one sample simultaneously (Assarsson et al. 2014). Furthermore, the assay is "homogeneous," which means that no washing steps are involved, the sample is simply mixed with the antibodies, and the signal is measured. As opposed to the heterogeneous ELISA assays, homogeneous assays are much less laborious; they are quicker and less prone to analytical confounders.

Finally, immunomagnetic reduction (IMR) assay has recently been developed by MagQu (MagQu Co. Ltd., Taiwan). This is again a homogeneous assay where magnetic nanoparticles coated with a biomarker-specific antibody are mixed with the sample and exposed to external magnetic fields, which forces the nanoparticles to align. Upon binding to a biomarker molecule, the nanoparticles become larger and consequently are slower to align with the field. The differences in the speed of alignment reflect the biomarker concentration. This novel assay has already been applied to measurements of human Aβ42 and Aβ40 (Chiu et al. 2012) and neuronal protein tau (Chiu et al. 2014) in plasma and showed correlations with the diagnosis of Alzheimer's disease and volumetric and cognitive characteristics of patients, respectively.

Despite the fast progress in immunological methods for biomarker detection, little progress has been made over the past 15 years toward a simple and definite dementia diagnostics. This is caused, in major part, by the lack of suitable, disease-specific biomarkers. Many immunoassays are based on the same set of antibodies, just applied to different technological platforms, improving analytical sensitivity but not specificity and selectivity of the assay. Therefore, the hunt is on for novel antibodies with suitable properties detecting other disease-associated (and disease-specific) epitopes on known proteins.

New biomarkers (disease-associated protein variants) are desperately sought for by applying the state-of-the-art proteomic techniques. It is expected that the biomarkers will arise from the better understanding of the pathophysiological processes underlying the progression of different forms of dementias and might thus provide not only improved diagnosis but also novel targets for disease-modifying therapies.

7.3 Proteomic Approaches in Biomarker Discovery

Identification of proteomic biomarkers related to any disorder means comparing the protein complements (proteomes) of normal and diseased tissues and selecting the proteins that are different. Those are candidate biomarkers, which need to be validated in disease models and population studies. It may sound like a straightforward procedure; however, this is exactly what hundreds of scientists were trying to do for cancer over the last more than 30 years with mixed success. The problems encountered are multiple. First, the biomarker for a given disease is usually present at a very low concentration, especially when the disease is in its initial stage and when a possible treatment is most effective. Second, identification of the biomarkers is dependent on the well-defined cohorts of patients and healthy individuals. Selection of such cohorts is often a difficult task, since the diagnostic methods for neurodegenerative diseases are not fully reliable (especially not in the early disease stages) (Beach et al. 2012). Third, the proteomic methods are themselves error prone, often introducing unintentional biases or false-positive results. Fourth, the proteins are present at highly differing amounts, and concentration ranges span up to 12 orders of magnitude. Fifth, the sheer number of various forms of proteins is staggering and overwhelms the capacity of any currently known separation and identification technique. The problem can be explained on neuronal protein tau, whose disease forms generate neurofibrillary pathology. The protein is coded by one gene, whose mRNA is alternatively spliced to produce at least six different protein isoforms. These isoforms are further modified by posttranslational modifications. More than 50 phosphorylation sites have been identified on the protein, along with several glycation, glycosylation, and acetylation sites. These modifications are present in various combinations. On top of these physiological modifications, tau undergoes hyperphosphorylation and multiple abnormal truncations in neurofibrillary pathology (reviewed in Kovacech and Novak 2010, Zilka et al. 2012). Tau, therefore, exists in tens or hundreds of different forms in normal brain, and this number is further multiplied in a diseased tissue. The same protein species scheme applies to all proteins, only differing in the type and number of modifications. These different protein varieties are expected to have (at least slightly) different biological activities. They were termed *proteoforms* by Kelleher's group, which denote highly related protein molecules arising from all combinatorial sources of variation giving rise to products arising from a single gene (Smith and Kelleher 2013). The sum of *proteoforms* of all proteins defines a specific state of a cell, tissue, or the whole organism. Proteomic approaches hence attempt to identify *proteoforms* differentially present in a diseased tissue.

7.3.1 Proteome Fractionation Techniques

Identification of the biomarkers is done by *mass spectrometry* (MS), the powerhorse of the proteomic discovery. Since MS technology has its limits as to the ability to resolve thousands of similar biomolecules, various protein separation and fractionation methods were designed to generate defined fractions of the proteomes to ease the identification in a mass spectrometer.

Liquid chromatography (LC) was the first of the modern separation techniques, which was developed in the early 1900s by the Russian botanist Mikhail S. Tswett. The method allows separation of large amounts of material based on proteins' hydrophobicity, size, charge properties, affinity to other proteins (e.g., antibodies), and presence of glycans or phosphogroups (Snyder et al. 2010). It is even easy to automate and standardize and allows sequential combination of two (even three) LC methods (reviewed in Dugo et al. (2008), Di Palma et al. (2012)). Its big advantage is a relatively straightforward interconnection to a mass spectrometer for the direct identification (or quantification) of the separated proteins or peptides.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffer (SDS-PAGE) is the second widely used protein separation technique. It has been invented by Laemmli in 1970 (Laemmli 1970). The SDS-denatured proteins are separated based on their size in a solid mesh of polymer filled with water and buffer molecules. The method was later updated to 2D electrophoresis, where proteins are first separated based on their charge and then (in the perpendicular direction) according to their size (O'Farrell 1975). The method was broadly applied to discovery proteomics (Oliveira et al. 2014).

Capillary zone electrophoresis (CE) is the newest from the classical proteomics techniques, although experimentation with this technique was documented already in 1930 by Tisselius (Petersen and Mohammad 2001). The proteins and peptides are separated in a thin capillary in an electric field based on their charge and size. The method is highly reproducible, fast, easily to automate, and highly sensitive with high resolution able to separate thousands of compounds (Stalmach et al. 2015). Furthermore, CE can be connected online to mass spectrometers, requires low sample volume, and is relatively cheap. The only disadvantage is the limited size of the proteins that can be effectively analyzed when coupled to a mass spectrometer (<20 kDa); therefore the protein mixture is usually digested with a protease and analyzed in the form of peptides (Pejchinovski et al. 2015).

Many other fractionation and enrichment techniques are used to simplify the complex proteomes, which include selective lysis of cells to preserve organelles and separate nuclei, membrane-associated proteins, and intra- and extracellular vesicles (synaptic vesicles, mitochondria, lysosomal and autosomal vesicles, exosomes from extracellular space, etc.) usually by differential centrifugation or isolation of protein complexes by using affinity resins (lectins, antibodies, etc.) (reviewed in Drissi et al. (2013)). Finally, in order to identify low abundant biomarkers or preserve those with limited half-life, it may be necessary to isolate the population of specific cells of interest, which may be performed by cell sorting in flow cytometry, by enrichment using immunomagnetic methods, and by microdissection (Altelaar and Heck (2012).

7.3.2 Mass Spectrometry

Mass spectrometry (MS) technology was initially developed by physicists for the measurement of the masses of atoms and lead, for example, to the discovery of isotopes (beginning of the twentieth century). In the last ~25 years, the biomarker discovery has been greatly facilitated by the quickly developing MS methods (the clinical applications were recently reviewed in Scherl 2015). This replaced the cumbersome methods of protein identification based on Edman N-terminal degradation, cloning, and antibody detection. Modern MS allows not only unequivocal identification of a protein but also its characterization including confirmation of the amino acid sequence and posttranslational modifications, i.e., identification of the individual *proteoforms*.

A mass spectrometer measures the mass to charge ratio (*m*/*z*) of an ionized molecule in an evacuated tube. Proteins, peptides, and organic molecules can be ionized by different methods, but two of them are particularly useful for proteomic application: electrospray (ESI) and matrix-assisted laser desorption (MALDI). The *m*/*z* of the ions is then measured either in time-of-flight, quadrupole, ion trap, or ion cyclotron resonance mass analyzers (Scherl 2015).

MS is generally used for two applications: identification of unknown proteins and quantification of known targets. In the earlier stages of MS development, only small molecules could be effectively measured by mass spectrometers, and so the proteomes were first digested with a protease with defined cleavage sites (e.g., trypsin, LysC, LysN, etc.) (Giansanti et al. 2016) to shorter peptides amenable to MS analysis. This is called the "bottom-up" approach, and it still remains the mainstream of MS proteomics in various approaches.

In the *targeted proteomic* approach, the proteins can be quantified by selecting the protein-specific peptides (also posttranslationally modified) resulting from a digestion with a protease. Upon ionization, the peptide is selected and subjected to collision-induced fragmentation, and then the resulting selected fragment ion(s) is quantified. By introducing a defined amount of isotopically labeled internal standard for the protein, it is even possible to quantify its *absolute* amount. Furthermore, hundreds of peptides (proteins) can be quantified in the sample in parallel, thereby capturing a fairly complex dynamics of proteomes of the cell/tissue/organ (Soste et al. 2014). The targeted proteomics becomes a method of choice for the quantification of clinically important proteins and peptides, steadily replacing the widespread ELISA methods due to its high precision, reproducibility, flexibility (no need for labeling or antibodies), and multiplexing. Methods for MS quantification of amyloid β proteins and tau in CSF have been developed and implemented along with an MS-based method for characterization of reference material used for standardization of ELISA testing of CSF (Korecka et al. 2014; Leinenbach et al. 2014; McAvoy et al. 2014; Portelius et al. 2015; Barthelemy et al. 2016; Pannee et al. 2016). The drawbacks of MS quantification include slightly lower sensitivity and need of a state-of-the-art instrumentation.

The *shotgun* proteomics is the mainstream bottom-up approach. It enables identification of thousands of proteins in complex samples and is usually applied in discovery-based projects. In this approach all peptides in the digested proteomes are

analyzed by a mass spectrometer. Depending on its speed, sensitivity, mass range, and resolution, the most abundant peptide ions are selected for fragmentation allowing the sequence confirmation and identification. The approach allows comparison of the protein/peptide abundance between two or more samples by labeling them with different isobaric tags, e.g., iTRAQ, SILAC, etc. (Rauniyar and Yates 2014), and thus identifies putative biomarkers. The abundance of peptides (and proteins) in the samples can even be compared by label-free quantification applying complex computational tools (Webb-Robertson et al. 2015).

The *bottom-up* approach is widely used for the analysis of posttranslational modifications of the proteins that could be associated with specific biological states of the cells or tissues. However, digestion of the proteins with a protease eliminates the possibility to compare the *proteoforms* of the expressed proteins between the two samples, because the specific pattern or combination of post-translational modifications is lost (Fig. 7.1), although some limited possibilities for analysis of proteoforms still exist (Lisitsa et al. 2014). Therefore, attempts are made to analyze the proteins in their intact forms, by the so-called *top-down* approach (Catherman et al. 2014).

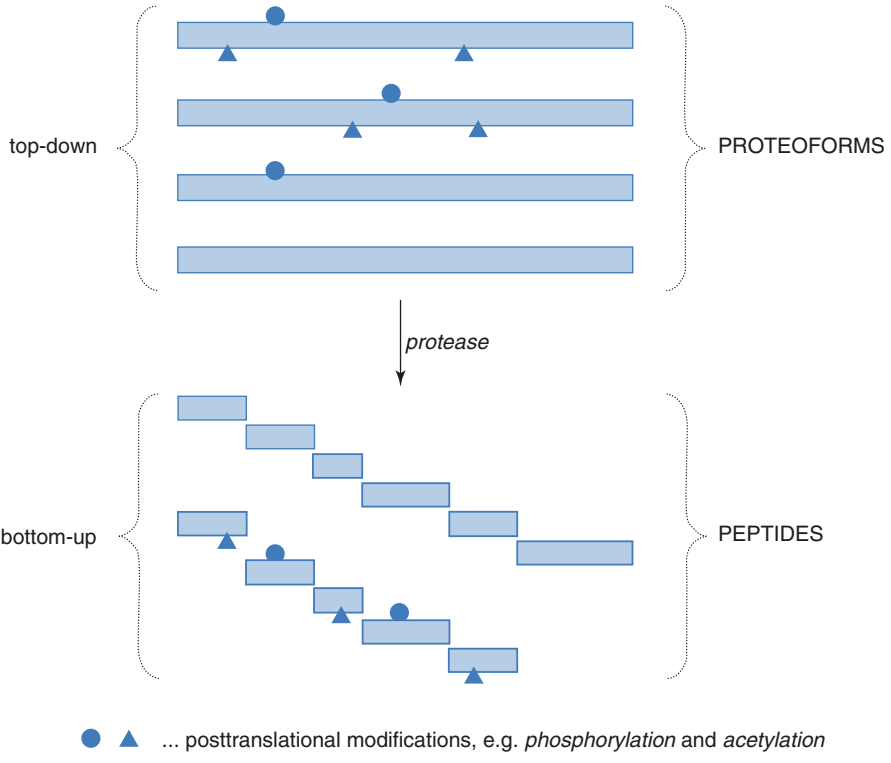

Fig. 7.1 Comparison of the top-down and bottom-up approaches in proteomics. Top-down approach preserves the proteoforms present in the sample, while digestion into peptides in the bottom-up approach degrades the proteoform information into a collection of subunits (peptides)

This approach poses a challenge for the MS instrumentation, since proteins larger than 20 kDa are difficult to analyze. When ionized by electrospray, the proteins attain multiple charges, which lead to "charge dilution" effect decreasing sensitivity. Furthermore, multiple charge forms of the proteins increase the chance that the ions of different proteins with the same m/z will overlap and hamper ion separation and characterization. To avoid such problems, the proteins need to be pre-fractionated and then the fractions introduced into a mass spectrometer. There are emerging techniques that solve these issues (Tran et al. 2011; Erba 2014; Molden and Garcia 2014; Sarsby et al. 2014; Scheffler 2014; Ye et al. 2014; Zhao et al. 2014; Guerrero et al. 2015) and allow label-free top-down quantitative comparison for biomarker development (Ntai et al. 2016).

The advances in proteomic methods and mass spectrometry instrumentation churning out systems with ever higher resolution, mass precision, robustness, and reproducibility move us slowly toward the complete characterization of the "protein complement" of the genome (Maurer et al. 2015; Wilson et al. 2015; Hu et al. 2016) and help us to understand physiological and pathological processes in the living organisms (Larance and Lamond 2015).

7.4 Summary

Biochemical markers related to the animal dementia are underdeveloped, despite the fact that dogs and cats provide natural models for human dementia. Biochemical diagnostics usually focuses on the body fluids as the most accessible sources of biological markers related to the disease. Cerebrospinal fluid and blood are the most interesting, since the former should contain the highest concentrations of the biomarkers and the latter is the easiest to collect. A number of immunoassays are in use for human dementia diagnosis that can be directly applied in canine and feline dementia diagnosis. Furthermore, dogs and cats provide excellent natural models of neurodegeneration and are therefore well suited for the identification of novel biomarkers using state-of-the-art proteomic methods.

Acknowledgment The authors received funding by APVV-0399-10, APVV-15-0613, and APVV-14-0872 and VEGA grants 2/0155/13 and 2/0164/16.

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