EARLY NONINVASIVE DIAGNOSIS OF NEURODEGENERATIVE DISEASES

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ABSTRACT
This paper reviews the contemporary trends in the pathobiochemistry of neurodegenerative disorders with respect to their early predictive diagnosis and possible treatment interventions. If we consider the current epidemiological data related to neurodegenerative disorders, medicine is going to face in the near future latent pandemic situations. The introduction puts an emphasis on the emerging importance of one major cluster of neurodegenerative disorders: diseases of the abnormal protein beta-conformation. The cluster includes such significant diseases as Alzheimer, Pick, Huntington, Parkinson disease, as well as the transmissible spongiform encephalopathies (Creuzfeldt-Jakob disease). The pathogenetic mechanisms in the determination of this group of disorders are explored with an emphasis on the impairment of post-synthetic chaperone correction. The central role of a number of such protein products is discussed. In particular the pathobiochemical mechanisms concerning the formation of beta-amyloid, alpha and beta synucleins, scrapie isoform of the prion protein are presented. A new diagnostic principle allowing the early and specific diagnosis of the conformation diseases protein via amplification techniques is presented. These methods compete in sensitivity with the PCR methods and shows promises for effective treatment. In conclusion, beta-pathies are considered a suitable example for the modern concept of cluster and prototype diagnosis in medicine and especially in clinical neurosciences.

Key words: neurodegenerative diseases (Alzheimer, Pick, Huntington, Parkinson disease), protein amplification techniques, synucleins, prionoses, beta-pandemics

INTRODUCTION
Neurodegenerative diseases (ND) are at the basis of one of the most destructive pandemics of the 21 century.¹² A central place among ND is occupied by the so-called beta-pathies — diseases characterized with spontaneous conversion of the alpha spirals of brain proteins into beta chains that are rigid, hydrophobic, and are prone to avalanche-like deposition in neurotoxic aggregates. Beta-pathies are not synonymous with prionopathies: they are a large cluster of neurodegenerative diseases and diseases of other systems in which the secondary conformation of protein molecules is impaired to the formation of rich in beta-chain polymers and prionopathies are a special case of the above. Table 1 presents the prevalence of major beta-pathies in the US and the UK.¹³ Due to the increase of life expectancy and the disturbed ecological balance (the global warming, the emergence of xenobiotics) these figures are estimated to increase four-fold by 2025 and eight-fold by 2050 as seen in the table. Thus, by 2050, the number of cases of beta-pathies worldwide will be 50–100 million people. The average annual cost of treating a patient with beta-pathy is $ 40,000 which means that the total cost for the entire world could amount to almost $ 2000 billion. This figure includes potential patients in many countries, including Bulgaria, about which there is no statistically reliable data yet.

Almost 90% of beta-pathies occur sporadically and in elder patients, while 8–9% are genetic and 1–2% with infectious genesis.² The latter two groups of beta-pathies feature an early debut - before the age of 50 and are usually of more severe course. As a whole, beta-pathies are irreversible diseases that result in disability and death. Because of their latent course early diagnosis and therapy at this stage are practically impossible. Epidemiological
methods of prevention of prionoses are exceptions to a certain extent.²

We believe that within the next 5-10 years we may experience a breakthrough in the counter measures against this potential pandemic as a result of the implementation of a series of clinical-laboratory and imaging methods, which are highly sensitive and noninvasive, and blaze a way to early diagnosis and treatment of beta-pathies. Particular emphasis will be put on the so-called immunologic amplification methods that already compete with the DNA and RNA polymerase chain reactions. Simultaneously, new imaging and biochemical methods are being introduced by which the disease process can be monitored in vivo and in situ.

PATHOBIOCHEMISTRY OF BETA-PATHIES

Major obstacle in the research and timely diagnosis of beta-pathies is their long “incubation” or long latency before manifestation, sometimes as long as decades. To challenge this, biological models of rodents, roundworms, and especially yeast are being developed in which the period is shortened to 10 days.

A key role in the pathogenesis of beta-pathies is probably played by the mechanism that disrupts the post-translational processes after the synthesis of alpha spirals and which reorganizes them in the form of beta chains, which are actually nuclei for the further avalanche-like multiplication. The formation of nuclei affects the polypeptide aggregates at the level of sub-cellular organization.

The alpha spiral is flexible and can be dissolved by proteases, which prevents it from aggregation. Beta-chain conformation in turn is rigid and insoluble, making it prone to aggregation (Fig. 1).

Table 1. Prion diseases and neurodegenerative diseases - prevalence in England and the USA in 21st century (after Prusiner)

<table>
<thead>
<tr>
<th>Disease</th>
<th>2001</th>
<th>2025</th>
<th>2050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prion diseases</td>
<td>150</td>
<td>4000</td>
<td>32 000</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>4 000 000</td>
<td>10 000 000</td>
<td>20 000 000</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>1 000 000</td>
<td>3 000 000</td>
<td>5 000 000</td>
</tr>
<tr>
<td>Frontotemporal dementia</td>
<td>40 000</td>
<td>80 000</td>
<td></td>
</tr>
<tr>
<td>Pick’s disease</td>
<td>5000</td>
<td>10 000</td>
<td>20 000</td>
</tr>
<tr>
<td>Progressive supranuclear palsy</td>
<td>15 000</td>
<td>44 000</td>
<td>100 000</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>20 000</td>
<td>40 000</td>
<td>100 000</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>30 000</td>
<td>60 000</td>
<td>100 000</td>
</tr>
<tr>
<td>Spinocerebellar ataxias</td>
<td>12 000</td>
<td>25 000</td>
<td>40 000</td>
</tr>
</tbody>
</table>

Figure 1. Transitions between secondary conformation states of proteins (modification by the authors after L. Sirakov, in Biochemistry, Gachev E. ed, St Kliment Ohridski University Sofia, 1995).
The question remains why this change in conformation is irreversible and leads to the formation of an increasing number of aggregates, plates and residual corpuscles, resulting in disability and inevitable death.

New evidence has been emerging recently that the basis of this mechanism is the quality control defects of brain proteins\textsuperscript{4,5} The quality control is implemented, similarly to our clinical laboratories, in three stages (Fig. 2):

- Presynthetic level of DNA
- Synthetic RNA level
- Postsynthetic protein level

Defects of the first stage are those of the counters of trinucleotide repeats. For example, in Huntington’s chorea the impaired “counters” allow surpluses of more than 40 impaired repeats of trinucleotide GAG coding the synthesis of the neurotoxic polyglutamate Huntington product. In the second stage defects the control of small interfering RNA declines, which reveals opportunities for early diagnosis and treatment of certain beta-pathies.

Defects of the third, postsynthetic stage, emerge to be the most important.\textsuperscript{6-8} In them the chaperones are the most affected - small peptides (heat-shock proteins, ubiquitins, protein X - in prionoses, parkin in Parkinsonism). In compliance with their names (from the French Governesses), they control the folding, organelle compartmentation and correcting of possible defects by refolding, selective removal, recycling and degradation in selective organelles (lysosomes, proteasomes). In damage to the selective degradation and turnover, they turn into the so-called residual corpuscles through sporadic “filling of the bins”.

An essential pathogenetic mechanism in the beta-pathies is the amplification of abnormal polypeptide products described by Soto in prionoses and recently confirmed in Alzheimer’s and Parkinson’s diseases. This problem is elaborated in detail in the subsequent sections of this review.

Beta-amyloid dimers tend to rise to oligomers which are converted into nuclei (hence nucleation) for irreversible polymerization. From the formed polymers blocks are separated (resembling blocks of melting iceberg), which are recycled as new centers of polymerization. A process of cyclic amplification occurs to facilitate ante-mortem diagnosis of some beta-pathies as prionoses and Alzheimer’s disease.

The accumulation of beta-peptides in the CNS impairs memory on the first place. As it is known, human memory is embedded in the form of engrams in nearly 1000 trillion synapses, which is more than 30 times the capacity of the library of the U.S. Congress and nearly 10,000 times the memory of a laptop. Memory flexibility is due to a rise in the number of neurons and synapses in mental work (one neuron accounts for about 500 to 1000 synapses). Coordinator of the different routes of memory is the hypo-campus amygadalae complex. It is assumed that the “center” of the actual memory is the hippocampus and of the emotional - the amygdaloid body (Fig. 3).

Higher mammals also have a flexible memory. For example, for the Bottlenose Dolphin used in the therapy of mentally impaired children any success of the kids expands their volume of memory and then rewards them with a smile by activating

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Impaired quality control of protein synthesis (illustrated by the authors\textsuperscript{4,5}).}
\end{figure}
the amygdaloid nucleus.

In Alzheimer’s the presenile hippocampus atrophy and that of adjacent brain lobes lead to dementia. A key factor for its emergence is the enzyme presenilin 1-gamma secretase that separates the beta-amyloid residue 42 from its BAPP precursor (beta-amyloid precursor protein).\textsuperscript{7,8} This enzyme is subject to regulation by a quintet of chaperons:

- The first two chaperones make coarse adjustment in terms of amplification (PS Enhancer) or weakening;
- The other chaperones provide the attunement, by balancing the operation of these chaperones to deliver optimum quantity of beta amyloid 42 (Fig. 4)

In mutations of genes regulating synthesis of Beta Amyloid Precursor Protein - as Down’s trisomy 21, the balance is disrupted leading to dementia. Similar events occur in almost 140 mutations that regulate the gamma secretase quintet and are responsible for the early manifestations of familial Alzheimer’s disease. Dementia deteriorates under the influence of two other proteins:

- \textit{tau} (a stabilizer of neurons’ cytoskeleton), which increases and hyperphosphorylates with an involvement of abnormal isoforms of kinase enzymes. Moreover, tau aggregates laterally in folded spiral threads that tangle the beta-amyloid and calcium ions associated with it. In turn the calcium ions activate the so-called lethal “calcium pump” and the ions of copper, iron and aluminum trigger oxidative stress. The cumulative effect leads to apoptosis, neuronolysis and dementia.\textsuperscript{8}
- The disease is exacerbated in the presence of epsilon 4 isoforms of ApoE which catalyzes the formation of amyloid plaques, resembling red carnations containing residues of neurofibres and bivalent cations.

In experimental mice, the \textit{tau} protein plays a crucial role for the onset of dementia, whilst amyloid plaques are less important. It is uncertain yet remains open whether this holds to be true in humans for the purposes of applying \textit{tau} inhibitors as possible therapeutic agents. Additional cofactors are brain injuries, alpha-synuclein, accumulation of

\textbf{Figure 3.} Functional neuromorphology of memory (after 32).
heavy metals in the brain (aluminum), and some pro-atherogenic influences. In line with these co-factors tertiary prevention with statins, calcium antagonists, aspirin, etc. is applied towards worsening of symptoms. Recently, encouraging results are obtained after application of beta-synuclein which inhibits aggregation of the alpha-synuclein.

PRINCIPLES OF EARLY DIAGNOSIS OF BETA-PATHIES

The laboratory diagnosis of Alzheimer’s disease is carried out in three aspects:

• Search for predictors such as trisomy 21 and the isoform of the epsilon 4 allele of ApoE. The studies of Siest et al. including our region, demonstrate that the increase of epsilon 4 allele, especially in homozygotes is often but not necessarily a predictor of the disease;

• Search of liquor markers, as increases of tau, especially of its hyperphosphorilation products as well as reduction of beta-amyloid 42, especially in its relationship with tau. In this respect, amplification techniques for direct determination of beta amyloid in serum of patients are being developed;

• Search for mutations affecting the gamma secretase quintet using DNA proteome methods for detection of familial predisposition, respectively for the individual treatment of the disease.

The most reliable diagnosis is performed with holistic approach that includes imaging methods in the following order: CT - MRI - PET (Positron Emission Tomography) – Pittsburgh Component - in PET.

A leading marker for Parkinson’s disease is increased synuclein alpha. Three main forms are distinguished in the family of synucleins:

1. Alpha synuclein stimulates presynaptic rearrangement as a chaperon, providing the processing of presynaptic group of SNARE proteins. It forms a bridge between Parkinson’s disease and Alzheimer’s and their mixed forms (dementia with Levi cells; supranuclear progressive paralysis, frontotemporal dementia of Pick, etc.) Normally alpha synuclein determines flexibility of synapses, protects synaptic terminals and regulates exchange of deoxyphenylalanine. Meanwhile it is isolated as non-A4 component of amyloid-precursor protein. Its effect on presynaptic rearrangement and formation of new neurons was demonstrated recently by observations in songbirds, where during the mating period pursuit of mastering the increasingly sophisticated melodies enhances synaptic rearrangement, especially among the winners of the singing competition.

2. Beta synuclein is a synergist of the alpha isoform regarding synaptic rearrangement. As mentioned previously, the levels of both synucleins increase repeatedly in songbirds in the mating period, especially in male canaries, thrushes and nightingales that are known to excel in singing. Similar changes in mammals (dolphins, primates, etc.) when they learn to perform more complex tasks suggest that the probable expression of synucleins in musically gifted children and composers might be of scientific interest.

Especially critical is the role of beta-synuclein
as a balance of the alpha-isoform aided by several chaperons, parkin for instance, which help to fine adjust the alpha-beta synuclein equilibrium.\textsuperscript{11,12} In its breach surplus alpha synucleins polymerise autocatalytically to insoluble fibrils which are deposited in the form of cells of Levi with other metabolic waste products (neuromelanin, ubiquitin, copper cations).\textsuperscript{13}

Very often the disease is triggered by:

- Viral infections – for instance the Spanish flu during the First World War took more victims than the war itself; it is possible that global warming may trigger similar “beta pandemics”;
- The toxic methylphenilpiridin is a component of many psychostimulants and insecticides, which directly affect negatively the dopaminergic neurons in a fast-developing Parkinson’s syndrome.
- Serious injuries, stress, etc. may also have a similar effect.\textsuperscript{14}

Currently the diagnosis of Parkinson’s disease is based on the search of beta synuclein isomers and of mutations in their encoding chaperons. As histochemical demonstration of Levi’s cells in the brain is an invasive procedure it is usually performed post mortem. Because of this minimally invasive approaches have been developed recently aimed at direct biochemical proof of the ratio alpha/beta synucleins in serum and cerebrospinal fluid for diagnosis of rare early (genetic) forms of the disease through amplification methods. In combined forms markers for Alzheimer’s are sought for, such as tau and beta amyloid 42. To assess the degree of neuronolysis proteins P-100-14-R 3-3 and neuron-specific enolase are examined. Diagnosis is supported by imaging studies.

Prionoses are the most dangerous beta-pathies affecting today more than 30 animal species from 52 countries, incl. Bulgaria, Greece, Macedonia, etc.. In humans, they are expressed mainly as Creutzfeldt-Jakob disease in three forms:\textsuperscript{2,15,16}

- sporadic - affects 90% of patients;
- hereditary - affects 10% and is due to mutations in the prion gene;
- infectious form - affects 1%, most often in relation to consumption of contaminated meat. Due to the prolonged “incubation” period a significant increase of this percentage is expected in the near future, which will repeat in a much more alarming scale the kuru epidemic from 1960-1970. (Gaydusek).

At this stage of our knowledge about prionoses the primary means to reduce this hazard is prevention\textsuperscript{2} which consists of:

- Prohibited use of bonemeal (prion vector) as animal feed for cattle, feed for domestic cats and a source for gelatin used for culinary and pharmaceutical products, namely capsules for the medicinal industry;
- Limiting the iatrogenic infections by disposable neurosurgical instruments and needles that are difficult to sterilize and also limiting the use of human pituitary gland for hormone extraction;
- Development of vaccination programs for populations at risk and prompt prophylaxis in the coming years.

Two critical areas are revealed in the prion molecule (Fig. 5). By the carbon terminal of the chain there are six octapeptide repeats, which show a strong affinity for the metal of neurosurgical instruments, and are therefore very difficult to disintegrate by it even at higher temperatures and in the regime of special treatment.\textsuperscript{8,17,18}

The most threatened by folding alfa spiral, which is within the scope of action of chaperon X, is located in the central segment of the prion molecule\textsuperscript{19,20}; near this area are to be found protective polymorphisms, protecting against folding of the chain (e.g. codons 129, 219) as well as risk mutations of which particularly dangerous are those at codon 200\textsuperscript{2,19}. It is estimated that in Slovenia and Libya, this mutation is responsible for 50 times greater prevalence of prionopathies among major population groups.\textsuperscript{1} It is very likely that this mutation can be found in countries of our region.

The report of Gambetta et al.\textsuperscript{21} for a specific form of Creutzfeldt-Jakob disease with pathogenic form of the soluble prion is of particular interest (in which the alpha spiral tertiary configuration is predominant). This opens a hypothetical perspective for the development of a reliable model for experimental therapy of the illness in its other forms.

The prion infection undergoes two stages:\textsuperscript{22,23} slow and fast. Its slow initial development in lymph nodes, tonsils, appendix and separate lymphocytes allows for in vivo diagnosis. In the second stage when the CNS is affected, the infection can be proven only by necropsy or biopsy examination of the brain.

Amplification methods, which principle is similar to polymerase chain reaction, give the most reliable results for very early diagnosis of prionoses.\textsuperscript{24-26}

The biological sample is incubated in a proton cell and is bombarded with ultrasound, which has the ability to accelerate the recycling of oligomer
units of the prion polymer. An increasing number of polymers is formed in this reaction which are amenable to nephelometric tracking. For 100 cycles Soto reached amplification of over 1 million times (Fig. 6).27,9

Similar results are obtained by two other immunobiochemical methods for signal amplification: the conformation dependent immunoassay (CDI) after Prusiner and SIFT (scanning of intensity of fluorescent targets after Eagen). Both authors are Nobel laureates.

**PMCA**

\[ \text{PMCA}^x = \text{prion amplification method} \]

\[ \text{PrP}^C + \text{PrP}^\text{Sc} \xrightarrow{x^{25}} (\text{PrP}^\text{Sc}) \]

\[ \text{NUCLEUS} \]

\[ \beta - \text{amyloid polymers} \]

\[ \text{y3} \]

\[ \text{140x recycling} \]

\[ \text{Immuno-nephelometry (10^x amplification)} \]

\[ ^x\text{PMCA} = \text{Protein misfolding cyclic amplification} \]

**Figure 5.** Structure of the prion molecule (after 2).

**Figure 6.** Amplification method (modified after Soto).
These methods allow for intravitral non-invasive diagnosis of prionoses in an infected animal (in the lymphoid tissue of the third eyelid) and humans (study of single lympho- and monocytes, as well as serum, urine and CSF). Transnasal biopsy of fila olfactoria terminalis seems to be of particular importance.

In its entirety amplification methods lay out the path to non-invasive diagnosis and treatment of prionoses and other beta-pathies through four approaches:

• Biochemical, including the described amplification and proteomic methods;
• Cytological (flow cytometry);
• Imaging methods (including so called PET for visualization of beta-chain isomers of the brain in vivo);
• Bio-tests on yeast and roundworms for rapid confirmatory diagnosis within days.

These approaches reveal new possibilities for etiological treatment using beta-breakers, alpha-stabilizers, manipulation of chaperons (thus PIB - Pittsburgh Compound-B) is an inhibitor of the X-chaperon, related to prionopathies), stem cells and their stimulation, synthesis of new vaccines.28

CONCLUSIONS

One of the major conclusions is that the large cluster of beta-pathies is an example of the validity of the principle of cluster prototypes and cluster diagnosis that was recently launched by several authors (K. Schaffner).29 As it turned out as a result of the accumulated experimental and laboratory data in biomedicine, and in particular in clinical neuroscience, the categorial principle of classification and diagnosis is depleted due to large areas of overlap of narrow diagnostic entities, such as Alzheimer’s disease.30,31 This overlap applies to clinical semiotics and, as shown in this review, to pathobiochemical determinants of diseases. That is why instead of narrow categories it is proceeded to introduction of evidence based expansion of diagnostic constructs to greater cognitive associations, such as clusters and prototypes. The contemporary 11th revision of ICD is based on this principle. In this context, it is the beta-pathies that represent a successful example of such evidence-based diagnostic group.

In addition, the reported diagnostic approaches change radically the prognosis for some diseases and provide opportunities for timely causal in vivo therapy.

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