Studies of *in vivo* prostate amyloidosis and autoimmune responses towards amyloid structures in neurodegeneration.

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Doctoral thesis

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Cover: Amyloid fibrils of S100A8/A9 (Photo: Kiran Yanamandra)

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Knowledge without action is useless;
Action without knowledge is foolish.

-Sathya Sai Baba

To my parents and family
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**Abbreviations**

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<th>Description</th>
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<tr>
<td>CA</td>
<td>Corpora amylacea</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
</tbody>
</table>
Abstract

By using multidisciplinary analysis of CA inclusions in prostate glands of patients diagnosed with prostate cancer, we have revealed that their major components are the amyloid forms of S100A8 and S100A9 proteins associated with numerous inflammatory conditions and types of cancer. We have demonstrated that material closely resembling CA can be produced from S100A8/A9 in vitro and shows the characters of amyloids. This process is facilitated by calcium or zinc, both of which are abundant in ex vivo inclusions. These observations were supported by computational analysis of the S100A8/A9 calcium-dependent aggregation propensity profiles. We have found DNA and proteins from Escherichia coli in CA bodies, suggesting that their formation is likely to be associated with bacterial infection. CA inclusions were also accompanied by the activation of macrophages and by an increase in the concentration of S100A8/A9 in the surrounding tissues, indicating inflammatory reactions. These findings, taken together, suggest a link between bacterial infection, inflammation and amyloid deposition of pro-inflammatory proteins S100A8/A9 in the prostate gland, such that a self-perpetuating cycle can be triggered and may increase the risk of malignancy in the ageing prostate.

We evaluated the autoimmune reactions to endocrine (insulin) and astrocytical (S100B) biomarkers in the blood sera of PD patients compared with healthy controls. Peripheral immune responses can be sensitive indicators of disease pathology. We found a statistically significant increase of the autoimmune responses to both antigens in patients compared with controls. Heterogeneity of the immune responses observed in patients may reflect the modulating effect of multiple variables associated with neurodegeneration and also changes in the basic mechanisms of individual autoimmune reactivity. We did not detect any pronounced immune reactions towards insulin amyloid fibrils and oligomers in patients, indicating that an amyloid-specific conformational epitope is not involved in immune recognition of this amyloid type. Immune reactions towards S100B and insulin may reflect the neurodegenerative brain damaging processes and impaired insulin homeostasis occurring in PD.

Generated auto-antibodies towards the major amyloidogenic protein involved in PD Lewy bodies - α-synuclein and its amyloid oligomers and fibrils were measured in the blood sera of early and late PD patients and controls by using ELISA, Western blot and Biacore surface plasmon resonance analyses. We found significantly higher antibody levels towards monomeric α-synuclein in the blood sera of PD patients compared to controls, though the responses decreased with PD progression. There were no noticeable immune responses towards amyloid oligomers, but substantially increased levels of IgGs towards α-synuclein amyloid fibrils both in PD patients and controls, which subsided with the disease progression. Pooled IgGs from PD patients and controls interacted also with amyloid fibrils of Aβ (1-40) and hen lysozyme, however the latter were recognized with lower affinity. This suggests that IgGs bind to amyloid conformational epitope, though displaying higher specificity towards human amyloid species associated with neurodegeneration. The findings suggest the protective role of autoimmunity in PD and therefore immune reactions towards PD major amyloid protein - α-synuclein can be used in treatment strategies and in diagnostics, especially in identifying early disease.

Key words: amyloid, amyloidosis, immune reactivity, S100A8/A9, insulin, α-synuclein.
**Introduction**

**Generic nature of amyloids**

The term amyloid was first introduced by German physician Rudolf Virchow in 1854. When Virchow examined the staining of cellulose-like material by iodine (Cohen & Jones 1991), for the first time he has transferred the designation of ‘amyloid’ from plant biology to a human brain structure called CA. Soon after this initial study, other diseased organs in the body were stained in the same manner and the amyloid term was used for these materials. In 1859 Friedreich and Kekule have demonstrated that the material which is stained in these organs are proteinaceous in nature (Kyle 2001). Consequently, the term amyloid was commonly used to describe the material which is deposited extracellularly in many organs. Since 1970’s the amyloids were defined as the fibrillar structures containing highly organized cross-β-sheet rich core, that was discovered by using methods of structural biology (Aoki *et al.* 1978, Eanes & Glenner 1968, Glenner 1980).

![Figure 1: Three dimensional structural models of amyloid fibrils. (a) Protofilament of Aβ peptide viewed down the long fibrillar axis (figure adopted from Tycko 2003). (b) Atomic structure of microcrystals assembled from GNNQQNY peptide (figure adopted from Nelson *et al.* 2005).](image-url)
Commonly, amyloid fibrils are long, un-branched and often twisted polymers of about ca. 10 nm in their diameter. Individual fibrillar strands or protofilaments are stabilized by inter and intra-molecular hydrogen bonds, hydrophobic and ion-pair interactions and composed of cross-β-sheets oriented parallel to, and the β-strands - perpendicular to the fibril axis (Figure 1a) (Eanes & Glenner 1968, Sunde & Blake 1998, Sunde et al. 1997, Tycko 2003). In cross-β-sheet motifs, the separation between hydrogen-bonded β-strands is ~ 0.48 nm, and the distance between β-sheet layers is ~ 1.0–1.3 nm (Figure 1b) (Serpell et al. 2000, Nelson et al. 2005).

Proteins, which usually fulfil specific functional roles, become inactive when they aggregate and form insoluble amyloid species such as fibrils (Bergstrom et al. 2005, Morozova-Roche et al. 2000, Pepys et al. 1993). A growing number of proteins and peptides have been shown to be able to form amyloids both \textit{in vivo} and \textit{in vitro}. They have no similarity in amino acid sequences and secondary or tertiary structures. However, when they self-assemble into the amyloid they share a common cross-β-sheet core. Based on the accumulated data, it has been suggested that the ability to form amyloid fibrils is an inherent property of polypeptide chains and most if not all polypeptides have ability to form amyloids, though their propensity to amyloid self-assembly can be different (Dobson 1999, Dobson 2001).

Protein undergoes amyloid assembly if they are partially destabilised or enter intermediate partially unfolded/folded states. A schematic presentation of some of the intermediate conformational states of the polypeptide chains and intermediate assemblies populated during amyloid formation are shown in figure 2 (Chiti & Dobson 2006). The process of amyloid formation can involve multiple fibrillation pathways.
Methods used to study amyloid structures

TEM and AFM methods (Chamberlain et al. 2000, Morozova-Roche et al. 2004, Morozova-Roche et al. 2000, Yanamandra et al. 2009) are widely used to study the morphology of the amyloids (Figure 3). The amyloids are characterized by inherent heterogeneity of oligomeric and fibrillar species, which can interconvert into each other during the course of protein incubation and amyloid assembly. The same high diversity of species is observed in ex vivo material, extracted from the human tissues. AFM has an advantage compared to TEM as it enables us to examine the amyloid structures both in air and liquid, in environment close to physiological condition; it does not require any chemical treatment of soft biological material, but it can be simply placed on the atomically flat mica surface. By using AFM the process of amyloid formation or disaggregation can be also monitored in real-time.
Figure 3: The amyloid fibrils of S100A8/A9 proteins involved in amyloidosis of aging prostate. TEM (a & b) and AFM (c & d) images (figure adopted from Yanamandra *et al.* 2009).

The amyloids in the histological tissues are primarily identified by using dyes such as Congo red and thioflavin T (*Satoskar et al.* 2007, *Westermark et al.* 1999, *Morozova-Roche et al.* 2000). The Congo red stained amyloid samples are viewed under polarized light where they are distinguished by a characteristic apple green birefringence. The samples stained with thioflavin T are characterized by intensive fluorescence in fluorescence microscope (Figure 4a and 4b). *In vitro* experiments, they are also often employed to identify amyloid and to monitor the kinetics of its formation.

Figure 4: Staining of amyloids. Congo red (a) and thioflavin T (b) staining of human lysozyme fibrils (figure adopted from Morozova-Roche *et al.* 2000).
The secondary diazo dye Congo red is schematically presented in figure 5. It has been suggested that it binds specifically to amyloids via insertion between the individual strands of cross-β-sheet (Carter & Chou 1998).

![Chemical structure of secondary diazo dye Congo red.](image)

Figure 5: Chemical structure of secondary diazo dye Congo red.

Thioflavin T is a benzothiazole dye and its fluorescence increases significantly upon binding to amyloid fibrils. Krebs and co-workers suggested that thioflavin T molecules bind to fibrils in the grooves on the face of the β-sheets that form the fibrillar backbone (Krebs et al. 2005)(Figure 6).

![Chemical structure of thioflavin T (a) and model of binding to amyloid fibril (b) (figure adopted from Krebs et al. 2005).](image)

Figure 6: Chemical structure of thioflavin T (a) and model of binding to amyloid fibril (b) (figure adopted from Krebs et al. 2005).

Immunohistochemistry is another method which is used to classify amyloids by using antibodies against known proteins involved in the amyloid formations as well as anti-fibrillar and anti-oligomeric antibodies. The amyloid assemblies display a generic conformational epitope, reflecting their common architecture, which is recognized by
conformation dependent antibodies (Kayed et al. 2007, Kayed et al. 2003, O’Nuallain & Wetzel 2002) (Figure 7). The sensitivity of this method is high to such an extent that the amyloids can be identified which escape the detection by Congo red or thioflavin T.

![Image of immunostaining of amyloid](image)

**Figure 7: Immunostaining of amyloid. Amyloids in the CA of ageing prostate was stained by using fibril specific antibodies (Kayed et al. 2007).**

**Amyloid diseases**

The hallmark of a wide range of human pathologies is the abnormal presence of extracellular protein deposits in a variety of organs or tissues including brain, different peripheral organs such as prostate, heart, spleen, liver etc. The protein components and location of the amyloid deposits in the body indicate the type of disease and its progression, but in all cases amyloids cause cells death and tissue damage (Table 1) (Caughey & Lansbury 2003, Dobson 2003, Siderowf & Stern 2003, Tan & Pepys 1994, Yanamandra et al. 2009, Chiti & Dobson 2006). To date more than 30 proteins with unrelated sequences and various secondary and tertiary structures are known to be implicated in protein misfolding and involved in human amyloid diseases (Table 1).

In local amyloidosis, proteins form amyloid deposits in a localized area and these amyloids are restricted to a particular organ or tissue. In systemic amyloidosis, amyloid deposits can be present throughout the body. Acquired amyloidosis is a complication of preexisting primary disease that produces either by inherently amyloidogenic abnormal
protein or greatly increased amounts of potentially amyloidogenic normal protein. Hereditary amyloidosis is caused by mutant genes encoding variant proteins whose structure makes them amyloidogenic. The amyloid formation in the brain is associated with AD and PD, Skelefteå disease, type II (non-insulin dependent) diabetes and others (Falk et al. 1997, Kahn et al. 1999, Martin 1999, Selkoe 1997). By contrast to others, in PD amyloid deposits of α-synuclein were found intracellularly (Croisier et al. 2005, Spillantini et al. 1998).

Table 1. Human amyloid disease associated with extracellular and intracellular deposits (adopted from Chiti & Dobson 2006).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating polypeptide</th>
<th>Structure of polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurodegenerative diseases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Amyloid β peptide</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Spongiform encephalopathy</td>
<td>Prion protein or fragments thereof</td>
<td>Natively unfolded (residues 1–120) and α-helical (residues 121–230)</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>α-Synuclein</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Dementia with Lewy bodies</td>
<td>α-Synuclein</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Superoxide dismutase 1</td>
<td>All-β, Ig like</td>
</tr>
<tr>
<td>Huntington's disease</td>
<td>Huntingtin with polyQ expansion</td>
<td>Largely natively unfolded</td>
</tr>
<tr>
<td>Spinocerebellar ataxia 17</td>
<td>TATA box-binding protein with polyQ expansion</td>
<td>α+β, TBP like (residues 159–339); unknown (residues 1–158)</td>
</tr>
<tr>
<td><strong>Non-neuropathic systemic amyloidoses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL amyloidosis</td>
<td>Immunoglobulin light chains or fragments</td>
<td>All-β, Ig like</td>
</tr>
<tr>
<td>AA amyloidosis</td>
<td>Fragments of serum</td>
<td>All-α, unknown fold</td>
</tr>
</tbody>
</table>
### Disease | Aggregating polypeptide | Structure of polypeptide
--- | --- | ---
Senile systemic-amyloidosis | Amyloid A protein
Familial amyloidotic-polyneuropathy | Wild-type transthyretin | All-β, prealbumin like
Hemodialysis-related amyloidosis | Mutants of transthyretin | All-β, prealbumin like
Apo amyloidosis | β2-microglobulin | All-β, Ig like
Lysozyme amyloidosis | N-terminal fragments of apolipoproteins | Natively unfolded
Fibrinogen amyloidosis | Mutants of lysozyme | α+β, lysozyme fold

**Non-neuropathic localized diseases**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating polypeptide</th>
<th>Structure of polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II diabetes</td>
<td>Amylin, also called islet amyloid polypeptide (IAPP)</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Injection-localized amyloidosis</td>
<td>Insulin</td>
<td>All-α</td>
</tr>
<tr>
<td>Corneal amyloidosis associated with trichiasis</td>
<td>Lactoferrin</td>
<td>α+β</td>
</tr>
<tr>
<td>Cataract</td>
<td>γ-Crystallins</td>
<td>All-β</td>
</tr>
<tr>
<td>Prostate corpora amylacea</td>
<td>S100A8/A9</td>
<td>All -α</td>
</tr>
</tbody>
</table>

During the last decade, a number of proteins has been discovered to form amyloids \textit{in vivo}, but these amyloids are not involved in any diseases. They show the physicochemical characteristics, dimensions and morphology of amyloids related in diseases. This indicates that nature utilize the amyloid formation for the beneficial function \textit{in vivo} (Table 2).
Table 2. Non-disease related amyloidogenic proteins (adopted from Chiti & Dobson 2006).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th><strong>Function of the resulting amyloid-like fibrils</strong></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curlin</td>
<td><em>Escherichia coli</em> (bacterium)</td>
<td>To colonize inert surfaces and mediate binding to host proteins</td>
<td>(Chapman <em>et al.</em> 2002)</td>
</tr>
<tr>
<td>Chaplins</td>
<td><em>Streptomyces coelicolor</em> (bacterium)</td>
<td>To lower the water surface tension and allow the development of aerial hyphae</td>
<td>(Claessen <em>et al.</em> 2003)</td>
</tr>
<tr>
<td>Hydrophobin EAS</td>
<td><em>Neurospora crassa</em> (fungus)</td>
<td>To lower the water surface tension and allow the development of aerial hyphae</td>
<td>(Mackay <em>et al.</em> 2001)</td>
</tr>
<tr>
<td>Proteins of the chorion of the eggshell</td>
<td><em>Bombyx mori</em> (silkworm)</td>
<td>To protect the oocyte and the developing embryo from a wide range of environmental hazards</td>
<td>(Iconomidou <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>Spidroin</td>
<td><em>Nephila edulis</em> (spider)</td>
<td>To form the silk fibers of the web</td>
<td>(Kenney <em>et al.</em> 2002)</td>
</tr>
<tr>
<td>Intralumenal domain of Pmel17</td>
<td><em>Homo sapiens</em></td>
<td>To form, inside melanosomes, fibrous striations upon which melanin granules form</td>
<td>(Berson <em>et al.</em> 2003)</td>
</tr>
<tr>
<td>Ure2p (prion)</td>
<td><em>Saccharomyces cerevisiae</em> (yeast)</td>
<td>To promote the uptake of poor nitrogen sources ([URE3])</td>
<td>(Chien <em>et al.</em> 2004)</td>
</tr>
<tr>
<td>Sup35p (prion)</td>
<td><em>Saccharomyces cerevisiae</em> (yeast)</td>
<td>To confer new phenotypes ([PSI+]) by facilitating the readthrough of stop codons on mRNA</td>
<td>(Eaglestone <em>et al.</em> 1999, True <em>et al.</em> 2004, True &amp; Lindquist 2000)</td>
</tr>
<tr>
<td>Rnq1p (prion)</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Not well understood ([RNQ+], also known as [PIN+], phenotype)</td>
<td>(Giaever <em>et al.</em> 2002)</td>
</tr>
<tr>
<td>Protein</td>
<td>Organism</td>
<td>Function of the resulting amyloid-like fibrils</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------------------</td>
<td>----------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>HET-s (prion)</td>
<td>Podospora anserina (fungus)</td>
<td>To trigger a complex programmed cell death phenomenon (heterokaryon incompatibility)</td>
<td>(Coustou et al. 1997, Saupe 2000)</td>
</tr>
<tr>
<td>Neuron-specific isoform of CPEB (prion)</td>
<td>Aplisia californica (marine snail)</td>
<td>To promote long-term maintenance of synaptic changes associated with memory storage</td>
<td>(Si et al. 2003)</td>
</tr>
</tbody>
</table>

**Kinetics of amyloid formation**

The kinetics of amyloid formation involves three phases - lag phase, elongation and finally plateau (Figure 8a). The initial lag phase is also called as neucleation phase. In this phase, proteins unfolds from their native states and form partially unfolded molecules which bind together and form nuclei. The time of the lag phase indicates how long it takes to form the nuclei. The formation of nuclei may take from seconds to days, depending on the protein and the environmental conditions. Once the nuclei are formed, then the process of fibrillar growth is rapidly increased either by association of monomers or oligomers to the nuclei (Jarrett & Lansbury 1993). The nucleated growth mechanism is well studied experimentally. It has been shown that certain changes in the experimental conditions like addition of preformed fibrils as seed to the beginning of reaction or certain types of mutations in the protein can reduce or completely abolish the lag phase (Figure 8b) (Merlini & Bellotti 2003). Immediately preceding after nucleation is the elongation phase. In the elongation phase, the nuclei are extended to produce well ordered protofilaments. The plateau or steady state phase corresponds to maximum of mature fibrillar growth and here no further polymerization occurs (Morozova-Roche et al. 2000). The time course of the conversion from native protein into fibrillar species can be monitored by thioflavin T fluorescent specific dye.
Figure 8: Kinetics of amyloid formation. (a) The schematic representation of the stages of amyloid formation (figure adopted from (Wilson et al. 2008)). (b) Seeded and non-seeded amyloid formation. The blue line indicates the formation of non-seeded amyloids and red line indicates the shortening of lag phase by adding pre-formed fibrils (figure adopted from Merlini & Bellotti 2003).

**Mechanisms of amyloid formation**

Different models of amyloid formation are based on the kinetic studies and includes template assisted (Griffith 1967), monomer-directed conversion (Prusiner 1982), nucleated polymerization (Lomakin et al. 1996, Jarrett & Lansbury 1993) and nucleated conformational conformation models (DePace et al. 1998, Serio et al. 2000).

In the template assisted model, peptides present in a soluble state (S) in random coil state bind to preassembled nuclei in a rapid pre-equilibrium state. This step is followed by a rate-determining structural change to add the peptide to the growing end of the fibril or filament, presumably as part of the β-sheet-rich quaternary structure (Figure 9a) (Griffith 1967). The monomer-directed conversion model suggests that a monomeric peptide can adopt a conformation called the ‘A’ state that is analogous to the conformation adopted in the fibril. The rate-determining step is that the ‘A’ structured monomer binding
and converts ‘S’ state monomer to an ‘A’ state dimer (Figure 9b). The formed ‘A’ state dimer then dissociates to an ‘A’ state monomers and they are rapidly added to the end of the growing fibril (Prusiner 1982). The nucleation polymerization model is characterized by the rate-limiting formation of a nucleus resulting from equilibrium between monomers that are and are not competent in forming assembly (Figure 9c). Once a nucleus is established, assembly occurs by the addition of assembly competent monomers to the growing end of the fibril (Jarrett & Lansbury 1993, Lomakin et al. 1996). The nucleated conformational conversion model contains the features of nucleation polymerization and template assisted models, as well as new features (Serio et al. 2000, DePace et al. 1998). In this model, nuclei are formed by accumulation and association of oligomers lacking a defined quaternary structure. Once nuclei are formed, they interact with structurally flexible oligomers with a distribution of subunits, adding a group of subunits to the fibril end (Figure 9d). Therefore, in this model, oligomeric complexes are crucial intermediates forming the amyloid nucleus. When these complexes undergo a conformational change on association with the nuclei, rapid fibrillar assembly follows (Serio et al. 2000). Each particular amyloid scenario need to be carefully investigated to conclude which specific mechanisms are involved.
Figure 9: Putative models of protein conversion into amyloid fibrils. (a) Templated assembly; (b) monomer-directed conversion; (c) nucleated polymerization; (d) nucleated conformational conversion. A-state denoted by smooth circles, rough circles represent S state (figure adopted from (Kelly 2000)).

Localized corpora amylacea amyloid deposits in ageing prostate

Recognition of prostate inclusions commonly denoted as CA and are found in a significant proportion in males over the age of 50-60 years and older have clinically importance. Prostate CAs have also been detected in 55% of cases in a study of high-grade prostatic intra-epithelial neoplasia in specimens derived from radical prostatectomy (Bostwick et al. 1993). CAs are tiny, calcified bodies of amyloid deposits, which grow up to few millimeters in diameter in the ageing prostate. The CAs are frequently observed
adjacent to the damaged epithelial cells and focal inflammatory infiltrates (Christian et al. 2005, De Marzo et al. 2007, Jager et al. 1997). They are often associated with the focal acute and chronic inflammation, epithelial trauma and gland occlusion (De Marzo et al. 2007). The CAs are asymptomatic and cause urinary retention and chronic inflammatory conditions like prostatitis or chronic pelvic pain syndrome (Bedir et al. 2005, Geramoutsos et al. 2004). There is a growing body of evidence indicating that inflammation plays a crucial role in prostate pathogenesis, as it is found to be associated with 40-90% of benign prostatic hyperplasia (Untergasser et al. 2005) as well as with 20% of all human cancers (De Marzo et al. 2007, Coussens & Werb 2002).

In spite of a high incidence of prostate CAs in late life (Cross et al. 1992), prostatic amyloidosis has not received sufficient attention. In several early studies it was reported that prostatic CA could contain amyloid structures (Cross et al. 1992, Gueft 1972, Marx et al. 1965); however these structures were also viewed as calcified bodies, prostatic concretion or calculi, resulting from calcification of precipitated prostatic secretion (Klimas et al. 1985) or arising from simple precipitation of salts normally presented in prostatic fluid (Drach et al. 1978). Localized amyloid deposits, which were not defined as CA or calcified inclusions, have been also described in prostate, seminal vesicles and in the lower urinary tract in some case studies on human patients by using clinical, radiological, MRI and immunohistopathological techniques (Jager et al. 1997, Jun et al. 2003, Singh et al. 2005). The protein content of prostate CA was investigated by immunohistochemical staining using a panel of antibodies against the major known amyloidogenic proteins (Cross et al. 1992, Rocken et al. 1996); β2-microglobulin was identified, but the antibodies to other proteins, which were not known as amyloidogenic thus far, have not been subjected to this examination. Besides this, the application of immunostaining as a sole method to detect proteins poses questions, especially if potential candidates may be present not in the native state, but in amyloid form. Therefore, in our studies we have evaluated the composition of CA and its mechanism of formation.

**Amyloid formation in Parkinson’s disease**

PD is the second most common progressive neurodegenerative disorder after AD, with a prevalence of 0.5%-1% among elderly population, rising up to 3% over the age of 80
The symptoms in PD include resting tremor, bradykinesia and muscular rigidity, which can become increasingly difficult to control as the disease advances (Fahn et al. 1998, Mayeux 2003). In PD, the clinical symptoms develop only after the death of 70-80% of dopaminergic neurons in the substantia nigra (Schapira 1999). They are often characterized by intracellular amyloid inclusions known as Lewy bodies.

The major fibrillar protein of Lewy bodies is α-synuclein, a normally unfolded protein that plays an essential role in synaptic transmission and synaptic plasticity, acting as presynaptic neurotransmitter (Liu et al. 2004). α-synuclein is present mostly in the cytosol where some of its fraction binds to the lipid membranes to form amphipathic helixes (Murphy et al. 2000, Clayton & George 1998). α-synuclein is relatively abundant in the brain under non-pathological conditions. There is substantial evidence that the conversion of α-synuclein from its soluble form to the aggregated insoluble form in the brain is a key event in the pathogenesis of PD (Dawson & Dawson 2003). In vitro α-synuclein can self-assemble to form ordered amyloid species which are characterized by cross-β-sheet structures, similar to the aggregates found in Lewy bodies and formed by many other proteins such as Aβ, lysozyme and others (Bennett 2005), suggesting that this protein is sufficient to form inclusions (Uversky 2003). The inclusion bodies are intracellular and upon the neurons death or damage of axons in the substantia nigra, they are released into the extracellular space. Recent studies showed that both monomeric and oligomeric forms of α-synuclein have been found in the CSF and serum of PD patient (El-Agnaf et al. 2003, El-Agnaf et al. 2006, Tokuda et al. 2006).

**Insulin and S100B in Parkinson’s disease**

It has been reported that there is a high prevalence of insulin resistance in PD patients and 50-80% of impaired glucose tolerance is also observed in PD patients (Sandyk 1993). The investigation of the National survey from 24,831 elderly adults filing medicare claims found higher rates of diabetes and hypertension in adults with PD than in people without PD (Pressley et al. 2003).
The homeostasis of insulin is altered in progression of the PD. Dopamine neurons and insulin receptors are densely present in the substantia nigra region of the brain (Unger et al. 1991). The dopamine neuron loss in the substantia nigra region of PD patients is accompanied by decreased reactivity of the insulin receptors and a depression of the relative level of insulin receptor mRNA (Moroo et al. 1994, Takahashi et al. 1996). Few human studies demonstrated that the PD patients, using some drugs like levodopa, exhibit hyperglycemia and hyperinsulinaemia, while other drugs like bromocriptine affected the insulin sensitivity in patients (Van Woert & Mueller 1971, Sirtori et al. 1972). Thus, neurotrophic effects resulting from the insulin/insulin receptor system have been recognized as important in determining the etiological basis of PD. It was known previously that senile dementia in Alzheimer’s type patients led to decreased blood sugar concentrations and elevated serum insulin levels (Bucht et al. 1983). In addition, animal models revealed that the insulin given intranasally, could be transported to hypothalamus and hippocampus without affecting the blood glucose and insulin concentrations (Fehm et al. 2000). These evidences support the view that insulin is able to cross the blood brain barrier under pathological conditions.

Calcium binding protein, S100B, is produced and released mostly by astrocytes in the CNS (Donato 2001). S100B is considered as a biomarker for the brain damage. In healthy people this protein has different functions such as cell cycle regulation, modulation of cytoskeleton proteins, differentiation, growth and mobility (Heizmann et al. 2002). The altered expression of S100B is seen in the pathological conditions such as neuronal injury, neurodegeneration, cancer and others (Heizmann et al. 2002, Harpio & Einarsson 2004, Unden et al. 2005). In addition, it was also known that the CSF levels of S100B has been significantly elevated in the patients with mild or moderate AD but declines to normal levels in more advanced stage of the disease (Peskind et al. 2001). Several other studies have been conducted with the aim of establishing S100B as a biomarker for neuronal dysfunction including PD (Hovsepyan et al. 2004, Schaf et al. 2005, Poletaev et al. 2000). Biphasic antibody levels to S100B were detected during distinctly diagnosed dementia stages in AD. The autoimmune responses to S100B and the S100B concentrations, matched moderate to severe dementia progression (Gruden et al. 2007). In this study, we have
evaluated the immune response towards S100B and insulin, in order to understand the PD pathology.

Results

Paper I

In this study we have applied multidisciplinary approach to characterize the prostate CA inclusions extracted as a result of prostatectomies. Our results have shown that there are presences of pro-inflammatory calcium-binding proteins such as S100A8, S100A9 and calprotectin - S100A8/A9 complex, which are considered to be the biomarkers for prostate cancer. Furthermore, we focused on the role of amyloid self-assembly of these proteins in the formation of calcified human deposits by extracting the proteins and amyloids from the CA.

In order to identify the protein composition, we have applied the samples with protein extractions on liquid chromatography coupled with electrospray ionisation mass spectrophotometer. In all the specimens S100A8 and S100A9 were consistently found. We also observed traces of other proteins including three bacterial proteins. Bacterial 16S rDNA detected in PCR belongs to Escherichia coli.

The extracts of CA were also subjected to SDS-PAGE and Western blot analysis. We found monomeric (14 kDa) and dimeric (28 kDa) species of S100A9. S100A8 antibodies recognized 10 kDa S100A8 monomer. Human serum albumin antibodies interacted with a ca. 50 kDa molecular species, indicating the presence of truncated serum albumin. However, anti-serum albumin antibodies did not recognize the high molecular weight aggregated species which remained in the stacking gels. They were stained by both S100A8 and S100A9 antibodies, indicating that these aggregates are composed of both these proteins.

Immunohistochemistry experiments also revealed that the CAs were positively stained with both S100A8 and S100A9 antibodies. In addition, CAs were positively stained by anti-amyloid fibril antibodies. However, anti-albumin antibodies showed weak staining at the edges of CA inclusions and in the surrounding tissues indicating that serum albumin, detected by mass-spectrometry and Western blot analysis, came from the surrounding
tissues, and not from the CA bodies. These results demonstrate that the amyloid in the CA inclusions are of S100A8/A9 proteins and constitutes the significant mass of the CA specimens.

AFM and TEM were used to examine the *ex vivo* CA extracts, and they revealed the presence of a variety of highly heterogeneous aggregates. The amyloidogenic properties of the S100A8/A9 proteins were further examined to produce their amyloid forms *in vitro*. The S100A8/A9 complexes, extracted from granulocytes and produced recombinantly from *Escherichia coli*, were each incubated under the native conditions of pH 7.4 and 37 °C with agitation and at pH 2.0 and 57°C without agitation. Under both conditions these proteins were found to assemble into heterogeneous fibrillar species. As these *ex vivo* CA deposits were found to be calcified and to contain zinc salts, we examined the amyloid formation by S100A8/A9 in presence of extra calcium and zinc. We have observed that in the presence of extra calcium and zinc amyloid formation of S100A8/A9 proteins were promoted.

Intrinsic aggregation propensity profiles (Pawar *et al.* 2005, Tartaglia *et al.* 2008) of monomeric S100A8 and S100A9 at both pH 7.0 and pH 2.0 were calculated. The overall aggregation scores for S100A8 are 0.76 at pH 7.0 and 0.77 at pH 2.0 and for S100A9, 1.04 and 0.65, respectively; the aggregation score of S100A9 in particular is similar to the aggregation scores of Aβ (1–40) and Aβ (1–42) peptides at pH 7.0, which are equal to 0.97 and 0.94, respectively. The aggregation propensities of S100A8 and S100A9 in the natively folded S100A8/A9 oligomeric complex were calculated and the scores were of 0.18 and 0.32, respectively, indicating that they were reduced significantly. This indicates that the aggregation-prone sequences are involved in the oligomeric interactions.

In order to determine the mineral components of CA inclusions, the powdered CA samples were subjected to XPS analysis. A typical XPS spectrum of CA reveals the presence of C, N, O, P, Ca, K, Zn and Mg atoms. By x-ray powder diffraction measurements, it was confirmed the existence of hydroxylapatite (Ca$_5$(PO$_4$)$_3$OH) and whitlockite (Ca$_2$(PO$_4$)$_3$) crystalline phases in CA samples. Consistent with the XPS analysis, the Fourier transform infrared spectrum of CA powder showed characteristic amide I–III bands corresponding to peptide bonds in the proteinaceous phase, and the broad bands corresponding to the vibration mode of the phosphate group in calcium phosphate.
The above mentioned results revealed that the major components of prostate CA are the amyloid forms of S100A8/A9 proteins. We found DNA and proteins from *Escherichia coli* in prostate CA bodies. Our findings suggest a link between bacterial infection, inflammation and amyloid deposition of S100A8/A9 proteins in the prostate gland, which triggers self-perpetuating cycle leading to CA growth (Yanamandra *et al.* 2009). The CA formation in prostate gland is considered as a risk factor of tumor development and requires stringent attention.

**Paper II**

In this study we investigated the immune reactivity towards insulin and its amyloids and S100B in sera of PD and healthy controls. We have used ELISA as the major tool to measure the autoimmune response towards these antigens. For S100B, we have observed almost same median values for both PD and control groups when compare in the statistical box plots. However, mean value of PD patients is 50% higher than the control group ($P < 0.05$). In addition, we did not observe any correlation in PD patients among the age, gender, duration of disease and Hoehn and Yahr score.

Immune response towards insulin in blood sera of PD patients reveals that there was a significantly higher autoimmune antibody in the PD patients compared to age matched healthy control. From the statistical box plots, the mean and median values were 40% and 70% increased in PD patients compared to controls. We did not find significant correlation among the age, duration of disease and Hoehn and Yahr score. However, we have found significant gender correlation, as male PD patients showed significantly increased immune response than the female group ($P = 0.029$).

The insulin amyloid formation was controlled by measuring thioflavin-T florescence and the morphology of the amyloids was examined by AFM. Insulin oligomers were produced both at pH 2.0 and pH 7.4. Amyloid fibrils of insulin were formed after 50 h of incubation at pH 2.0. AFM analysis demonstrated that fibrils preformed at pH 2.0 remained stable in PBS under ELISA conditions. Consequently, immune response towards insulin oligomers and fibrils were screened in the blood sera of PD patients and controls. We did not observe immune reaction towards insulin oligomers and fibrils by ELISA in both PD
patients and controls, indicating that these antigens most likely are not present in the sera. (Wilhelm et al. 2007).

Paper III

There is significant evidence that the conversion of α-synuclein from soluble form to the aggregated insoluble form in the brain is a key event in the pathogenesis of PD. In this study we have evaluated the autoimmune response towards α-synuclein and its amyloid structures in the blood sera of PD patients. Firstly we have characterized the amyloid species of α-synuclein by using AFM and thioflavin T binding and cytotoxicity assay.

In order to examine the presence of auto-antibodies against α-synuclein monomeric species in the peripheral blood sera of each PD patient and healthy control individuals, we have applied ELISA and immunoblot detection methods. The results of ELISA analysis demonstrate that in healthy individuals the immune responses towards α-synuclein were at the cut-off level of ELISA, displaying a very narrow distribution of titers. By contrast, in early PD patients there was a significant increase of IgGs reactivity towards α-synuclein ($P < 0.0001$), accounting for rise by ca. 8 fold of mean and median values of titers compared to controls. In the blood sera of late PD patients there was also an increase of immune-reactivity towards α-synuclein compared to controls ($P < 0.007$), with ca. 6 fold higher mean and ca. 4 fold higher median, respectively, and with ca. 58% patients displaying high immune reactivity, but the values of their titers were lower than in the early PD group.

The analysis of immune-reactivity in the blood sera of patients and controls by immunoblotting demonstrate the same tendency as the results obtained by using ELISA: early PD patients are characterized by a significant increase in IgGs reactivity towards α-synuclein compared to controls ($P < 0.0001$) with ca. 5 fold and ca. 10 fold increase of mean and median values, respectively, as estimated by the density of Western blot bands, and with 63% of individuals exhibiting the high responses. In the late PD group the immune responses subsided, showing ca. 4 and 6 fold enhancement in mean and median, respectively ($P < 0.007$), compared to controls and with ca. 58% of patients showing high level of antibodies.
To provide an additional support to our findings we have subjected the pooled purified IgGs from blood sera of controls, early and late PD patients to Biacore surface plasmon resonance analysis. Consistently with the ELISA and immunoblot data, the level of IgGs binding to α-synuclein was significantly higher both in the blood sera of early \( (P < 0.0001) \) and late \( (P < 0.05) \) PD patients compared to controls, however, late PD group showed decreased level of antibodies compared to early PD patients.

Blood sera from early and late PD patients as well as healthy controls were also screened for the presence of immune response towards α-synuclein oligomers and fibrils. The autoimmune responses against α-synuclein oligomeric species did not reveal any statistically significant levels neither by ELISA nor immunoblot examination.

We have also screened the autoimmune reactivity against fibrillar species in the early and late PD patient and control blood sera by immunoblot analysis. There were no statistically significant differences in distributions of immune reactivities between the groups. The amyloid fibrils of α-synuclein were also used as antigens in the Biacore experiments and their interactions with the IgGs purified from the pooled blood sera of each studied group were monitored. There was significant immune reactivity towards fibrillar antigens in all samples with the slight decrease within 10% between the control and PD groups \( (P < 0.0001) \). This is consistent with the trend of decreasing immune reactivity towards amyloid fibrils with progression of PD detected by the Western blot analysis.

The purified IgGs from pooled blood sera from the PD patient and control groups were compared with regards to their reactivity towards fibrillar antigens of different protein origin, i.e. formed from α-synuclein, Aβ peptide and hen egg white lysozyme. Specific anti-fibrillar IgGs (Kayed et al. 2007), used as a reference, recognized all types of selected fibrils, but not corresponding monomers. By contrast the pooled IgGs of PD patients and controls interacted with the fibrillar antigens of all selected polypeptides, though their reactivity with α-synuclein and Aβ peptide fibrils was much more pronounced than with the fibrils of hen egg white lysozyme. Consistent with the above reported results the pooled IgGs from PD patients and in smaller extent from the controls recognized monomeric α-synuclein, but not the monomers of Aβ peptide or hen egg white lysozyme. This indicates that in blood sera of both PD patients and controls there are polyclonal IgGs
reactive with conformational epitope of amyloid fibrils, targeting, however, primarily the fibrils associated with human neurodegenerative ailments (α-synuclein and Aβ peptide).

**Conclusions of the articles**

In paper 1, by using a range of biochemical, biophysical and immunohistochemical methods we have showed that prostate CAs are composed of calcified proteinaceous amyloid deposits of pro-inflammatory proteins S100A8 and S100A9. Here for the first time we have shown that S100A8/A9 form amyloids, which are characterized by very distinct morphology – very thick bundles. The amyloid fibrils extracted from ex vivo material of CA were matched with the amyloids produced from S100A8/S100A9 under *in vitro* conditions. We have also showed that Ca and Zn, abundant in CA inclusions, induce the thickening of S100A8/A9 fibrils, which can contribute to their overall stability. The experimental observations were supported by computational analysis of the S100A8/A9 calcium-dependent aggregation propensity profiles. We found DNA and proteins from Escherichia coli in CA bodies, suggesting that their formation is likely to be associated with bacterial infection. CA inclusions were also accompanied by the activation of macrophages and by an increase in the concentration of S100A8/A9 in the surrounding tissues, indicating inflammatory reactions. Our findings, taken together, suggest a link between bacterial infection, inflammation and amyloid deposition of pro-inflammatory proteins S100A8/A9 in the prostate gland, such that a self-perpetuating cycle can be triggered and may increase the risk of malignancy in the ageing prostate. The results provide strong support for the prediction that the generic ability of polypeptide chains to convert into amyloids could lead to their involvement in an increasing number of otherwise apparently unrelated diseases, particularly those associated with ageing.

In paper 2, we evaluated the autoimmune reactions to endocrine (insulin) and astrocytical (S100B) biomarkers in the blood sera of 26 PD patients compared with controls by using ELISA. We found a statistically significant increase of the autoimmune responses to both antigens in PD patients compared with controls with a mean increase of 70% and 50% in the autoimmune reactions towards insulin and S100B, respectively. Heterogeneity of the immune responses observed in patients may reflect the modulating
effect of multiple variables associated with neurodegeneration and also changes in the
basic mechanisms of individual autoimmune reactivity. We did not detect any pronounced
immune reactions towards insulin amyloid fibrils and oligomers in PD patients, indicating
that an amyloid-specific conformational epitope is not involved in immune recognition of
this amyloid type, while sequential epitope of native insulin is hidden within the amyloid
structures. Immune reactions towards S100B and insulin may reflect the
neurodegenerative brain damaging processes and impaired insulin homeostasis occurring
in PD.

In paper 3, generated auto-antibodies towards the major amyloidogenic protein
involved in PD Lewy bodies - α-synuclein and its amyloid oligomers and fibrils were
measured in the blood sera of early and late PD patients and controls by using ELISA,
Western blot and Biacore surface plasmon resonance analyses. We found significantly
higher antibody levels towards monomeric α-synuclein in the blood sera of PD patients
compared to controls, though the responses decreased with PD progression ($P < 0.0001$).
This indicates potential protective role of autoimmunity in maintaining the body
homeostasis and clearing protein species whose disbalance may lead to amyloid assembly.
There were no significant immune responses towards amyloid oligomers, but substantially
increased levels of IgGs towards α-synuclein amyloid fibrils both in PD patients and
controls, which subsided with the disease progression ($P < 0.0001$). Pooled IgGs from PD
patients and controls interacted also with amyloid fibrils of Aβ (1-40) and hen lysozyme,
however the latter were recognized with lower affinity. This suggests that IgGs bind to
amyloid conformational epitope, though displaying higher specificity towards human
amyloid species associated with neurodegeneration. The findings suggest the protective
role of autoimmunity in PD and therefore immune reactions towards PD major amyloid
protein - α-synuclein can be used in treatment strategies and in diagnostics, especially in
identifying early disease.
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“The end of knowledge is love. 
The end of education is character.”

– Sathya Sai Baba

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References


