EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

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MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

GUT IN PRION AND PRION-LIKE DISEASE

Trabalho submetido por
Morgane Victoria Tomé
para a obtenção do grau de Mestre em Ciências Farmacêuticas

julho de 2024

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Trabalho orientado por
Professor Doutor Jorge Fonseca

e coorientado por
Profª Doutora Carla Ascenso

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To my mother Ana Lemos, that passed away, I hope this work sheds light on the hardship you endured. May you rest in peace, and may your story live on through this work and others that will come.

Acknowledgments

A massive thank you to my supervisors Professors Jorge da Fonseca and Carla Ascenso, whom despite being sceptic of the subject I chose were tireless in their support and help throughout this journey. If you read this, thank you for all you did beyond the supervision of my final graduation work, for your comprehension and your friendship. This work would have never seen the light of day without you.

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To all of the people mentioned here by name and those who will recognize themselves by reading this, thank you. To every single one of you, I am grateful for you, and I surely will never forget you.

And finally, thank you mom, I miss you. I hope I made you proud.

Resumo

As doenças causadas por priões foram descritas muito antes da descoberta dos priões. A primeira descrição de priões propriamente dita, relativa a "novas partículas infeciosas proteicas", só surgiu em 1982, num artigo escrito por Stanley B. Prusiner. Desde então, numerosos avanços na investigação permitiram compreender melhor os mecanismos adotados por estas doenças. Além disso, nos últimos anos, cada vez mais evidências apontam para a existência de mecanismos "prion-like" adotados por proteinopatias neurodegenerativas como as doenças de Parkinson e de Alzheimer. Com o crescente número de semelhanças encontradas entre as doenças causadas por priões e o aumento da literatura relativa à importância do eixo intestino-cérebro para as mesmas, esta revisão teve como objetivo reunir e sintetizar a literatura existente relativa à influência do intestino e dos seus tecidos e microbioma associados no prognóstico, desenvolvimento e potencial tratamento destas patologias. Esta revisão foi efetuada de forma exaustiva para englobar o maior número possível de evidências. Foram selecionados 102 artigos com base na sua relevância. Os resultados destacam os mecanismos de absorção de priões/proteínas desdobradas, a sua degradação no trato gastrointestinal e o envolvimento do sistema nervoso entérico e do tecido linfoide associado ao intestino na patogénese da doença. Esta revisão destacou a associação entre os processos gastrointestinais e o desenvolvimento de doenças neurodegenerativas, oferecendo pistas para guiar investigações futuras e para a descoberta de novos alvos e estratégias terapêuticas.

Abstract

The term "prion disease" was first used in 1923, long before the discovery of prions. The first description of prions as "novel proteinaceous infectious particles" was published in 1982 by Stanley B. Prusiner. Since then, numerous advances in research have allowed for a better understanding of the mechanisms adopted by these diseases. Furthermore, recent evidence indicates that neurodegenerative proteinopathies, such as Parkinson’s and Alzheimer’s disease, may adopt prion-like mechanisms. With the increasing number of commonalities between these diseases and the growing body of literature on the importance of the gut-brain axis, this review aimed to gather the existing literature on the influence of the gut and its associated tissues and microbiome on the prognosis, development and potential treatment of these pathologies. This review was conducted in a comprehensive manner to encompass the most evidence possible. A total of 102 articles were selected based on their relevance. The results highlight the mechanisms of prion/misfolded protein uptake, their degradation in the gastrointestinal tract, and the involvement of the enteric nervous system and gut-associated lymphoid tissue in disease pathogenesis. This review contributes to elucidating the relationship between gastrointestinal processes and the pathogenesis of neurodegenerative diseases. The findings are anticipated to inform future research and facilitate the development of novel therapeutic strategies.

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List of Abbreviatures

- AD: Alzheimer's disease
- BMDC: Bone marrow-derived cells
- BSE: Bovine Spongiform Encephalopathy;
- CNS: Central Nervous System;
- CWD: Chronic wasting disease:
- CXCR: Chemokine receptor;
- DC: Dendritic Cells;
- ENS: Enteric Nervous System;
- FAE: Follicle-Associated Epithelium;
- FDC: Follicular Dendritic Cells;
- FFI: Fatal Familial Insomnia;
- GALT: Gut Associated Lymphoid Tissue
- GPI: Glycophosphatidylinositol;
- LTi cells: Lymphoid Tissue inducer cells;
- M: Methionine;
- NK: Natural Killer
- NMDA: N-methyl-D-aspartate;
- NMDAR: N-methyl-D-aspartate (NMDA) Receptor;
- NPC: Neural precursor cells
- NSC: Neural stem cells;
- PD: Parkinson's disease;
- PIRIBS: Parallel In Register Intermolecular β-Sheet structure;
- PP: Peyer's Patches;
- PNS: Peripheral Nervous System;
- PRNP: Gene coding for the Prion Protein;
- PrP^{c}: Cellular Prion Protein;
- PrP^{Sc}: Scrapie-associated prion protein, more commonly used as a general abbreviation for disease-associated prion protein.
- REM sleep: Rapid-eye-movement sleep;
- sCJD: sporadic Creutzfeldt-Jacob Disease;
- sFI: sporadic Fatal Insomnia;

- STI-1: Stress-Inducible protein;
- TSE: Transmissible spongiform encephalopathies;
- vCJD: variant Creutzfeldt-Jacob Disease;

Glossary

- **Akinetic Mutism:** The condition is characterised by the presence of an intact level of consciousness and sensorimotor capacity, yet simultaneously exhibiting a decrease in goal-directed behaviour and emotions. The patients are in a state of profound apathy, seemingly indifferent to pain, thirst, or hunger. It represents the extreme end of the spectrum of disorders of diminished motivation (1).
- **Ataxia:** neurological sign manifested by a lack of movement coordination, caused by abnormal control of muscle contraction leading to gait abnormalities, changes in speech, and abnormal eye movements, also referred to as nystagmus (2).
- **Dendritic Cells:** Dendritic cells are **CD11c**$^{+}$ cells that link the innate and adaptive immune responses, via recruitment of immune effectors cells such as Natural Killer cells, and neutrophils, they are also characterized by their ability to migrate to target sites and secondary lymphoid tissues such as the GALT (3–5).
- **Extrapyramidal Symptoms:** a variety of movement phenotypes has since been described along the EPS spectrum, including dystonia, akathisia, and parkinsonism, which occur more acutely, as well as more chronic manifestations of tardive akathisia and tardive dyskinesia (6).
- **Goblet cells:** "Goblet cells arise from pluripotent stem cells and derive their name from their goblet, cup-like appearance". The primary function of goblet cells is to create a protective mucus layer standing between the gut lumen and its lining, they are also thought to be involved with immunoregulation (7).
- **Myoclonus**: sudden and brief involuntary muscle spasms, that cannot be controlled by the person experiencing it (8).
- **Pyramidal Symptoms:** lesions to the pyramidal tract can result in a range of debilitating consequences, including spasticity, hyperactive reflexes, weakness, and the Babinski sign (stroking the sole of the foot causes the big toe to move upward). These symptoms are all indicative of an upper motor neuron lesion. Damage to the corticobulbar tract may present with additional symptoms, including lower facial weakness and changes to speech (9).
- **REM sleep:** is characterized by rapid-eye-movements produced by the contraction of oculomotor muscles and other defining features such as a waking-like electroencephalogram pattern, active suppression of skeletal muscle activity (atonia). This sleep pattern is often short and preceded by non-REM sleep, followed by

wakefulness in healthy adults. Finally, REM sleep is also characterized by the presence of dreams (10).

- **Single Point Mutations:** The substitution of a single nucleotide in the DNA sequence with another nucleotide, which may or may not have a pathogenic effect (11)
- **STOP Codon Mutations:** stop codons are nucleotide triplets in mRNA that serve an important role in signalling the end of protein-coding sequencing process. Often, stop codon mutations will make a stop codon appear prematurely, meaning it is prior to its normal position in the gene. This results in a synthesized protein that is incomplete (or truncated) (12).

I. Introduction

Prion diseases were first described long before the discovery of prions. Although the first description of prion disease was Scrapie, in 1732 (13,14), the first description of prions as such, pertaining to "novel proteinaceous infectious particles", only came to be in 1982 in an article written by Stanley B. Prusiner (15). In this context, prions became the first ever infectious particles that did not contain genetic material from which they could replicate. It was later discovered that prions have an endogenous form named cellular Prion Protein (PrP^{c}), and that its disease-associated form, designated scrapie-associated Prion Protein (PrP^{Sc}), shares the same amino acid sequence. Nevertheless, these strains differ primarily in their conformation and, consequently, their biochemical properties, including proteinase resistance and solubility (16). Following the description of prion properties, researchers were able to define prion-caused diseases, which are more commonly referred to as transmissible spongiform encephalopathies (TSE) (13,16).

I.1. Prion protein structure-function relationship

The cellular prion protein is encoded by the single-copy PRNP gene, which is located on the short arm of chromosome 20 in humans (17–19). In 2019, Baral et al., in accordance with scientific consensus, stated that "the cellular prion protein is expressed almost ubiquitously throughout the human bodily tissues", nonetheless it has higher expression levels in the central and peripheral nervous systems (20–23). Examples of prion protein expression sites include a variety of neural cells such as neurons, astrocytes, oligodendrocytes, and microglia. PrP^{c} expression has also been reported in the Peripheral Nervous System (PNS) in both sensory and motor axons and Schwann cells (23). Outside the nervous system, PrP^{c} is also found in numerous other cell types, such as immune cells (i.e., immune cells, including T lymphocytes, natural killer cells, and mast cells), and is expressed in several major organs, such as the heart, pancreas, intestine, spleen, liver, and kidneys (23,24). Additionally, Prion Protein is expressed in different species including many mammals, other vertebrates such as fish, amphibians, or reptiles, and even some fungi and yeasts (18), with varying degrees of similarity (25,26). Despite being extensively studied, the function of PrP^{c} remains elusive to the scientific community (24).

- Primary to Quaternary Structure of the Prion protein

The primary structure of a protein is determined by its amino acid sequence (27). Prion protein is a 32-kDa glycophosphatidylinositol (GPI) -anchored glycoprotein precursor composed of 253 residues (18,28,29) as shown in Figure 1.

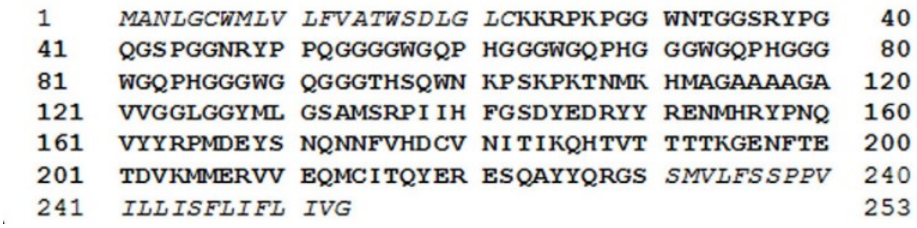

Figure 1: Primary structure of the cellular prion protein according to Uniprot entry P04156, the mature version of the protein is seen in bold.

After synthesis, PrP^c undergoes several post-translational changes until it reaches its functional form of 208 or 209 amino acids depending on the isoform, one being a membrane-bound glycoprotein and the other a cytosolic soluble protein, respectively (18,23,28,29). A major part of the precursor polypeptide is translocated to the endoplasmic reticulum by the N-terminal signal peptide where the C-terminal signal peptide is replaced by a GPI-anchor (24). This anchor allows PrP^c to be expressed as a cell surface protein (18).

The secondary structure of PrP^c is composed of two main domains (23,30): a N -terminal domain that is highly flexible as characterized by its random coil appearance, potentially facilitating interactions with other proteins, and a C-terminal domain that possesses a typical globular structure (Figure 2). The N -terminal domain contains four tandem repeats of an eight amino acids sequence, also called the octa-repeat region, which has been proven to interact with metal ions such as zinc and copper, in addition to a variety of other proteins. Interestingly, these findings have not yet been related to the specific function of PrP^c (21,30,31). The C-terminal domain is organized in specific secondary structures, including: three α-helices folded around each other to produce a three-helix bundle; two short antiparallel β-strands flanking helix 1; a disulfide bond connecting cysteines 179 and 214, linking helices 2 and 3; and two N-glycans on residues 181 and 197 (Figure 3) (21,23,32).

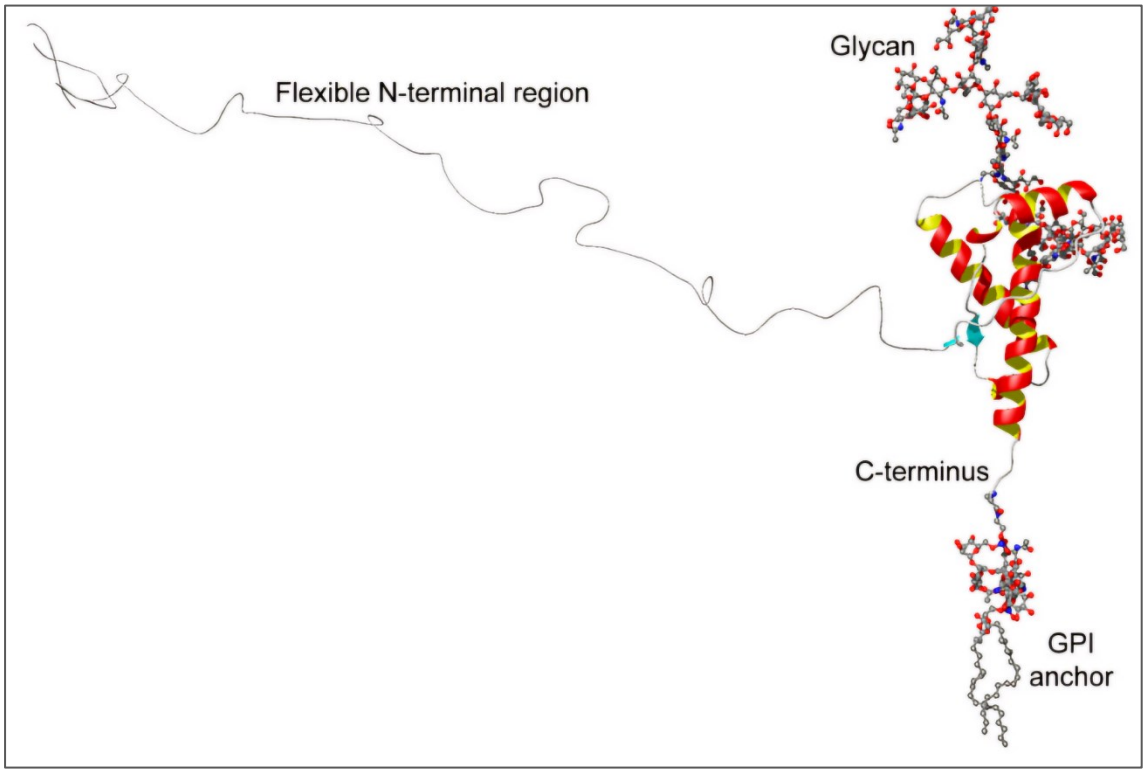

Figure 2: Ribbon diagram of the PrPc molecule. The C-terminal domain contains three α-helices, shown in red and yellow, and two β-strands shown in turquoise, whereas the N-terminal domain has been added on in a "random" configuration (24).

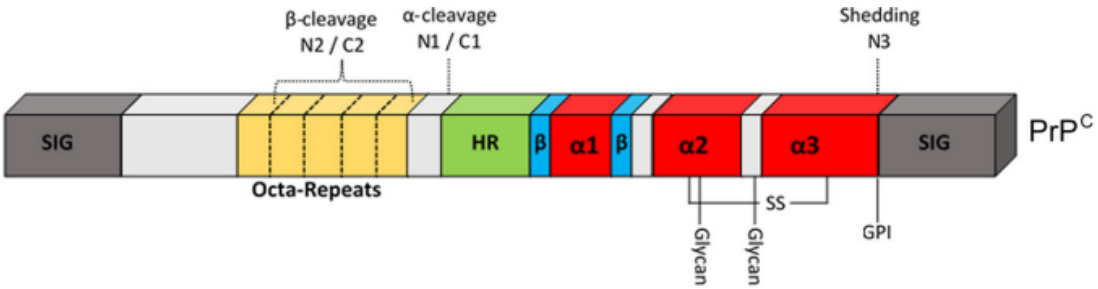

Figure 3: Schematic representation of PrP^c highlighting key structural features in greater detail (24).

The tertiary structure of a protein is described by its overall 3D shape, which is generated by the interaction between the amino acids of the secondary structures. PrP^c is mainly organised on the C-terminal side as a globular protein, whereas random coils characterise the N-terminal side with no specific structure (23,33). As for the quaternary structure, not all proteins possess one, as this type of organization requires at least two subunits, each containing a primary, a secondary, and a tertiary structure, closely interconnected to form a larger unit. These subunits are held together by hydrogen bonds and van der Waals forces between nonpolar side chains (34). Until recently, the hypothesis that PrP^c could organize itself as a dimer (two subunits forming a quaternary structure) was still only a theory, as previously outlined in the 2018 edition of the Handbook of Clinical Neurology, Vol. 153 (23). However, using X-ray diffraction methodology, in 2021, Bortot et al. (28) uncovered two different types of dimerization named α1 and α3 dimers, respectively, as a reference to the helix involved in the dimerization process. These advances in the understanding of PrP^c folding and polymerisation are significant because facilitate a more profound understanding of its structure *in vivo*. Moreover, provide insights into the functions of this protein and the processes involved in its misfolding (21,24,28).

- Functions of PrP^c

To date, the precise functions of prion protein have remained elusive to the scientific community (21,35). Despite this, throughout the years, scientists have been able to define several functions and the potential involvement of PrP^c in different mechanisms of protection and disease (35–39).

PrP^c during the Development of the Nervous System

In mammals, PrP^c becomes detectable during embryonic development's late stages, specifically during the neural tube's growth. Its expression then increases significantly shortly after birth (40). Although PrP^c has been proven non-essential for ensuring successful embryonic development, the literature strongly suggests that PRNP has a non-redundant function in maintaining cell pluripotency and differentiation during embryogenesis (41). One hypothesis defining the involvement of PrP^c in these early stages of development qualifies it as a receptor accompanying embryonic cells through neural differentiation and migration. This hypothesis was explained by the ability of some adhesion proteins, such as the laminin-integrin system, to recognize PrP^c (40,42).

Adhesion proteins, particularly integrins, are known to be important in embryogenesis and hold functions in neural precursor cells (43). Furthermore, in the absence of PrP^c, the interactions between the cell membrane and laminin-integrin system are impaired, resulting in abnormalities involved in cell differentiation, particularly affecting neuritogenesis and axonal growth (40). In this regard, despite not being an obligatory protein, PrP^c seems to be involved not only in the maintenance of neural stem cells (NSC) stemness and self--renewal, but also in the promotion of NSC differentiation into mature neurons in a dose-dependent manner (40,44).

PrP^c in the immune system

As previously mentioned, PrP^c is ubiquitously expressed throughout various human cell types (20). One of these cell types constitutes the immune tissues, such as T lymphocytes, natural killer (NK) cells, macrophages, dendritic cells (DC), regulatory T cells, and follicular dendritic cells (FDC) (45). However, once again, the exact role of PrP^c remains undiscovered. Studies have found that this protein plays a role in cell differentiation and maturation − a role in the embryogenesis of the nervous system, as previously referred to – exhibiting, for example, increased expression during NK cell differentiation and maturation (40,45). This phenomenon linking immune cell maturation and activation with elevated expression of PrP^c is also observed in T lymphocytes and dendritic cells (45). Furthermore, studies using PrP^c knockout ($Prnp^{0/0}$) mice, and mouse-adapted-Scrapie (ME7) infected mice, have revealed that this protein plays a role in the formation and preservation of secondary lymphoid tissues, such as the spleen and lymph nodes (45–47). Additionally, PrP^c seems to be involved in the correct development of the spleen lymphoid region, also called the white pulp, and the maintenance of normal levels of CD4 T cells and lymphoid tissue inducer cells (LTi cells) (45,46,48). Furthermore, it is interesting to highlight that of all lymphoid cells, PrP^c is expressed at higher levels in LTi cells, which are primordial to the formation of Peyer's Patches (PPs) (depicted in figure 4) and peripheral lymph nodes during embryogenesis (48–50). This fact, as discussed later, the disease-associated Prion Protein (PrP^{Sc}) travels from the bloodstream to the secondary lymphoid tissues, such as the lymph nodes, tonsils, PPs, and spleen, to utilize these cells as accumulation and replication sites (45). Studies on the participation of PrP^c in inflammatory responses have found that PrP^c is expressed at higher levels in immune-privileged organs (protected against inflammation), such as the brain, eyes, and

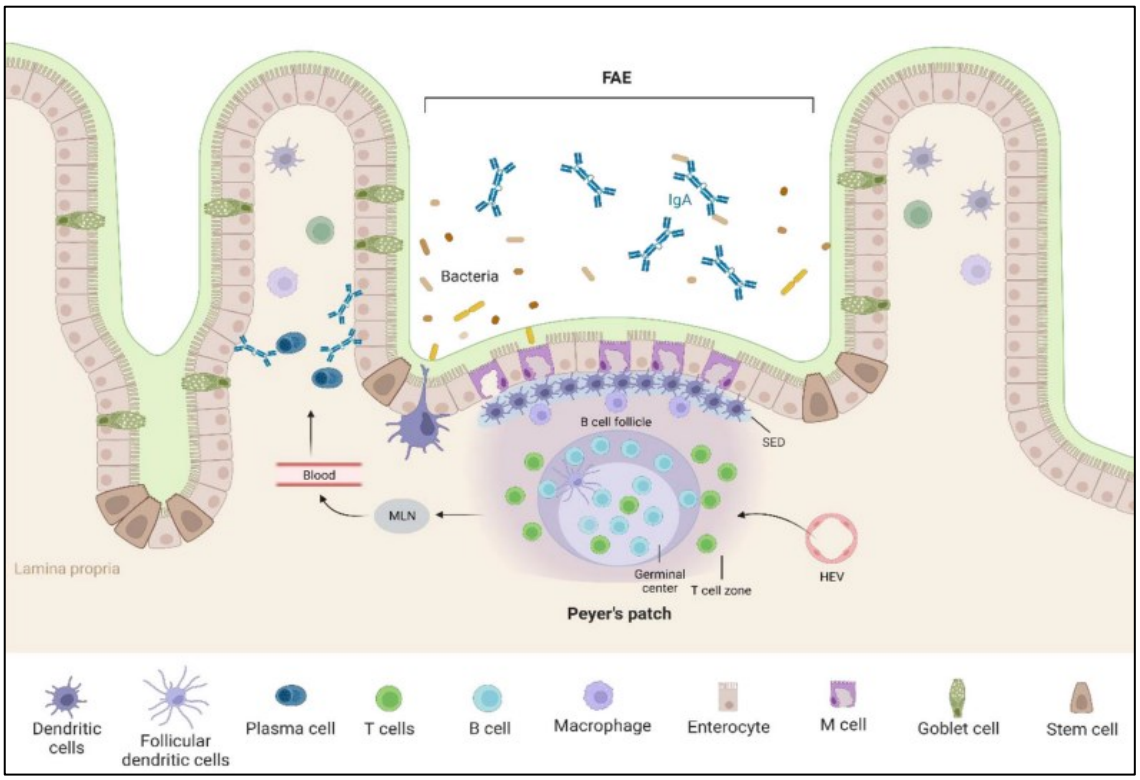

Figure 4: Intestinal PP structure with functional regions and constituting cells, from "Intestinal Peyer's Patches: Structure, Function, and In Vitro Modelling" (211).

both male and female genital organs. Furthermore, it appears that PrPc protects these organs from inflammation-derived damage through immunomodulatory processes. (45). Furthermore, the cellular Prion Protein has been shown to interact with the N-methyl-D-aspartate (NMDA) receptor, which is known to bind to glutamate, the principal excitatory neurotransmitter in the human brain (51,52). In addition, NMDA receptors (NMDAR) contribute to inflammatory pain and the transmission of pain signals in the spinal cord (51,53). It has been shown that PrP^c modulates the inflammatory response by inhibiting NMDAR, and decreasing pain sensation, whether neuropathic or nociceptive (51).

PrP^c and its protective role in the central and peripheral nervous systems

Several functions attributed to PrP^c can be seen as "neuroprotective". For example, several studies have found that PrP^c decreases cellular damage after ischemic stroke (21,31,37,39). This protective role seems to be partly explained by the idea that PrP^c acts as an antioxidant molecule and is therefore able to reduce ischemic damage by binding Reactive Oxygen Species (ROS), which are known to be implicated in this type of brain injury (21,54,55). In these cases, it has been shown that mice lacking PrP^c showed

more ischemic damage and less tissue repair. In contrast, wild-type mice (meaning they are able to synthesize PrP^c) showed that after ischemic injury under oxidative stress conditions (situation where concentrations of ROS are above normal), PrP mRNA levels increase, implying that these conditions upregulate PrP^c expression to decrease tissue damage (21,38,54). Moreover, PrP^c seems to inhibit NMDARs from blocking calcium ion (Ca^{2+}) cellular influx, thereby decreasing excitotoxicity, which is characterised by an exacerbated activation of glutamate receptors (such as NMDAR) that initiate a cascade of neurotoxicity, leading to neuronal cell death (21,56–58) Another mechanism by which PrP^c seems to exert its neuroprotective role is by reducing the phosphorylation of extracellular signal-regulated kinase (ERK1/2), which is known to regulate inflammatory responses, cytokines, and cell apoptosis in ischemic brain injury. The activation of ERK-1/2 can increase cell damage caused by ischemic injury by causing further oxidative stress and hypoxia-inducible factor 1α (HIF-1α) degradation (59–61). Nevertheless, other studies have indicated a correlation between PrP^c and the upregulation of the ERK1/2

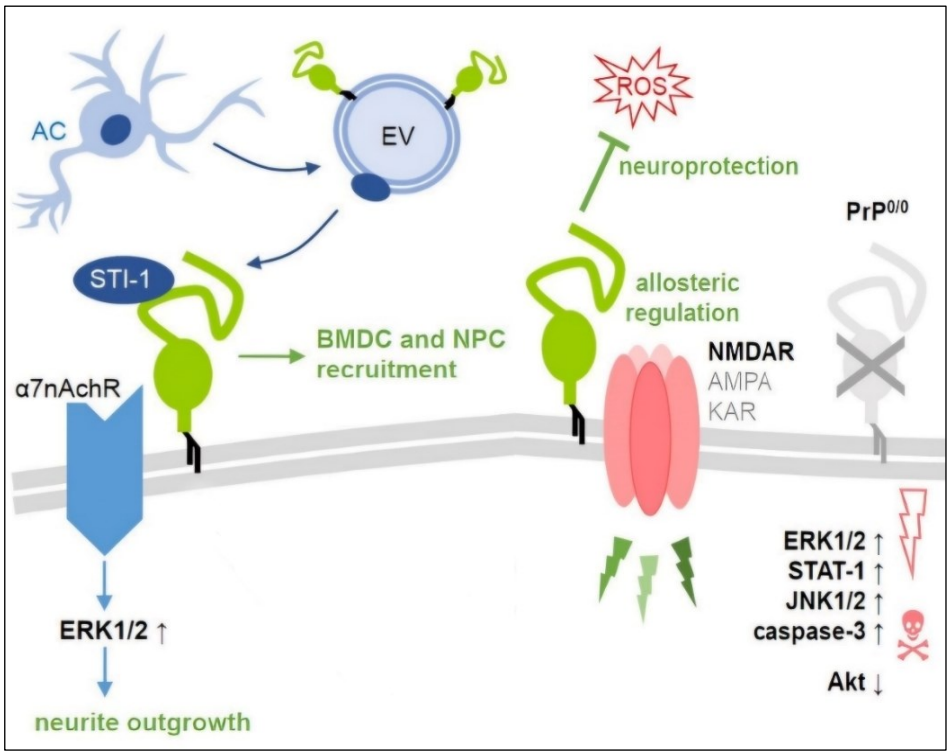

Figure 5: From left to right: the depiction of signalling pathways impacted by PrP^c after ischemic brain damage. First, we see ERK-1/2 upregulation (inhibition not shown) Consequences of this interaction may include recruitment of bone marrow-derived cells (BMDC) and neural precursor cells (NPC) leading to neurite outgrowth. Second, we see inhibition of NMDAR impeding excitotoxicity, and the protection of cells from ROS. Third, in the far-right example, we can observe the pathways that are activated when PrP^c is absent. Abbreviations and definitions: (JNK) Jun N-terminal kinase causes cell apoptosis; (STAT-1) signal transducer and activator of transcription 1, is implicated in neuroinflammation (212) caspase-3 involved in the apoptosis pathway (56); (Akt) Phosphoinositide 3-kinase/Akt signal pathway promotes neuronal survival (213). Adapted from (57).

pathway following ischemic insult. This appears to occur as a consequence of a direct interaction with Stress-Inducible Protein 1 (STI1), causing PrP^c endocytosis and modulating STI1-dependent ERK1/2 signalling involved in neuritogenesis, neuron survival, neurite outgrowth, and neuroprotection (21,24,38,62,63). Furthermore, overexpression of PrP^c has been demonstrated to reduce lesion size following ischemic stroke compared to wild-type mice. (31,64). The molecular pathways described above are summarized in Figure 5.

Regarding the expression of PrP^c in the Peripheral Nervous System (PNS) and its protective role, one of the most established functions of the cellular prion protein is the regulation of peripheral myelin maintenance and homeostasis (21). This is further proved by experiments done using $Prnp^{0/0}$ mice since, in these studies, knockout mice develop late-onset peripheral neuropathy characterized by demyelination, indicating that peripheral myelin maintenance is a bona fide function of PrP^c (31,56). PrP^c therefore regulates myelin homeostasis by interacting with G protein-coupled receptors (GPCR) expressed in Schwann cells called Gpr126, also known as Adgrg6, which triggers a signalling cascade that promotes myelination (Figure 6) (21,39,65,66).

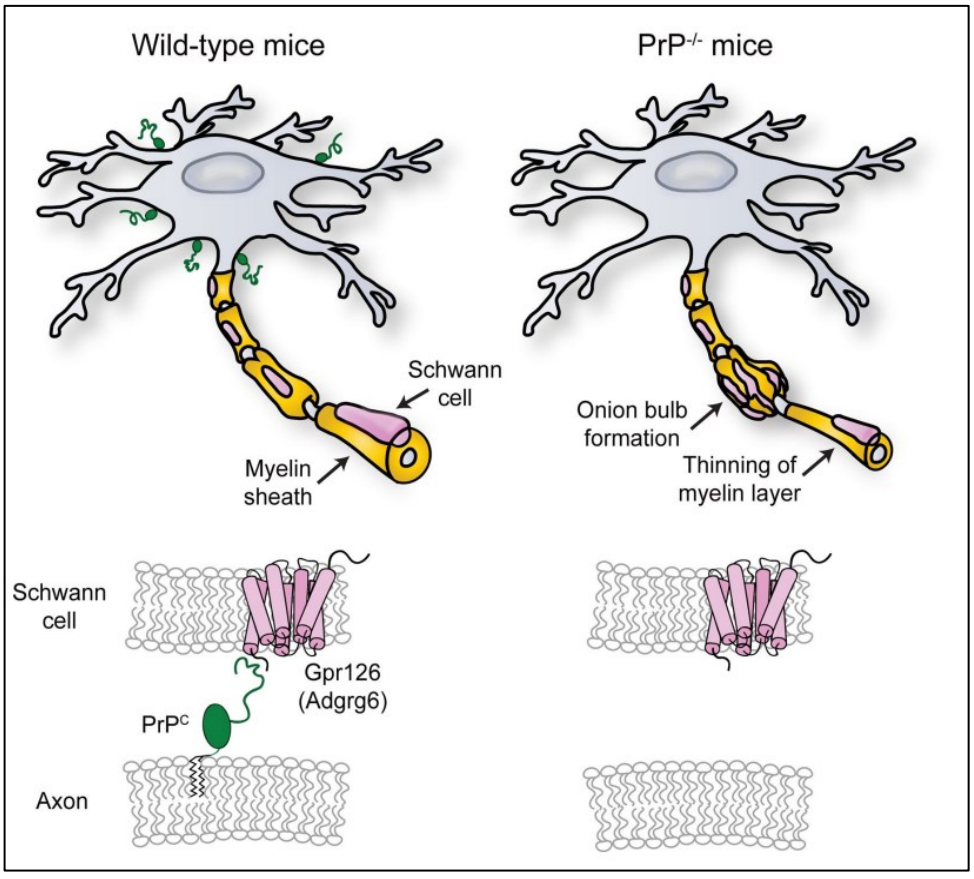

Figure 6: Process of myelin homeostasis with PrP^c on the left and chronic demyelinating polyneuropathy caused by the absence of PrP^c (39).

PrP^c and its role in the intestinal barrier

PrP^c is involved in cell-to-cell adhesion processes during the development of the CNS (40,42). However, this process has been identified in other cell types, namely enterocytes, which are the cells that constitute the intestinal wall. These cells perform several functions, including acting as a barrier between the intestinal lumen and blood circulation and facilitating the absorption of nutrients (67–69). What is interesting to point out is that some studies have found that decreased levels of PrP^c in enterocytes cause the cell-to-cell adhesion to weaken, therefore increasing the intestinal wall's permeability by increasing paracellular passage (67,70). These results demonstrate that PrP^c protects the organism from external aggression. However, it also could be a facilitating factor if infectious prions were ingested orally (71).

Other relevant functions of PrP^c

Despite the lack of clarity regarding the specific functions of PrP^c, this protein has been shown to influence some other mechanisms within the human body. For example, PrP^c is thought to play a role in cancer proliferation, particularly solid cancers such as pancreatic, colorectal cancer and gliomas, because of its overexpression in these types of cancer. Moreover, numerous PrP^c identified or potential partners are involved in signalling pathways known to modulate cell proliferation, which can determine cancer invasion, metastasis, and cell death. These pathways have been identified in human cancer cells (40,56). In recent years, an increasing amount of evidence points to the potential role of PrP^c in other neurodegenerative diseases such as alpha-synucleinopathies (Parkinson's Disease being the most common) and tauopathies (Alzheimer's Disease being the most prevalent) (56,72). As it happens, PrP^c has been found colocalized with Amyloid β-containing senile plaques of Alzheimer's Disease (AD) patients. Nevertheless, studies have yet to determine whether PrP^c plays a role in protection or as a potentiator for neurodegeneration (24,38). In the case of Parkinson's disease (PD), the degree of colocalization with PrP^c is lower. Despite this, in this disease, the elevated level of oxidatively modified proteins represents a significant contributing factor to the impairment of several cellular functions. As previously mentioned, PrP^c has the potential to act as a protector against oxidative stress (21,38).

I.2. Infectious Prion protein known mechanisms

As previously stated, the cellular Prion Protein has an isoform associated with disease, known as the Scrapie-associated Prion Protein, PrP^{Sc} (there are other diseases caused by misfolding of the prion protein, but for the sake of simplicity, this terminology is commonly used to refer to disease-associated Prion Proteins in general) (73). PrP^{Sc} is the first and only infectious particle with no genetic material. Consequently, it has been the subject of extensive research to further understand the underlying mechanisms of its propagation and transmission since its discovery approximately forty years ago (74). The objective of this section is to provide a comprehensive overview of the currently understood mechanism by which PrP^{c} undergoes misfolding into PrP^{Sc} and how this misfolded protein can result in the development of an infectious disease. The amount of PrP^{Sc} necessary for the infection of a new host (or the development of the sporadic disease) to be successful is very low (in the low femtogram range: 10^{-15} grams), yet the prevalence of these diseases in humans is extremely rare (75). Furthermore, although humans are susceptible to Bovine Spongiform Encephalopathy (BSE), the prion disease affecting bovine cattle, our species appears to be immune to Scrapie (affecting sheep) and Chronic Wasting Disease (CWD) affecting cervid populations (76).

- <u>PrP misfolding</u>

The Prion Protein must be misfolded to cause disease, whether it is sporadic/idiopathic (meaning of unknown aetiology) or acquired by infection. Until recently, the mechanism by which a normal protein misfolds into PrP^{Sc} remained a significant unresolved issue within the scientific community (77,78). In 2021, two papers were published on the mechanism by which the "initial" PrP^{Sc} is formed in a healthy individual, namely by Sanz-Hernández et al. and Kraus et al. Despite extensive investigate, protein conversion into PrP^{Sc} has remained unclear, particularly when considering a *de novo* situation. In their study, Sanz-Hernández et al. identified an intermediate between PrP^{c} and PrP^{Sc}, named $huPrP^{*}$ (77).

The intermediate form of prion protein was found to cause the aggregation of a specific strain of disease associated with Prion Protein, designated PrP mutant T183A. These findings, although specific to this mutant PrP^{Sc}, help to elucidate the mechanisms of conversion into pathological aggregates (Figure 7) (77). Nonetheless, similar to the huPrP* intermediate provided by Sanz-Hernández and coworkers in 2021, Kraus et al.

describe, in the same year, an intermediate in which the structure β-strand 1- α-helix 1- β-strand 2, is separated from α-helices 2 and 3, the latter being then able to untwist and form β-sheets. Furthermore, the β1 - β2 sheet has to be disrupted, probably generating another intermediate to break the intramolecular hydrogen bonds that will become intermolecular in PrP^{Sc} (Figures 8 and 9) (78). Once the misfolding process is complete, the PrP^{Sc} isoform is a β-sheet-rich protein with little to no α-helices. It also forms ordered aggregates, more commonly known as amyloid fibrils (78,79).

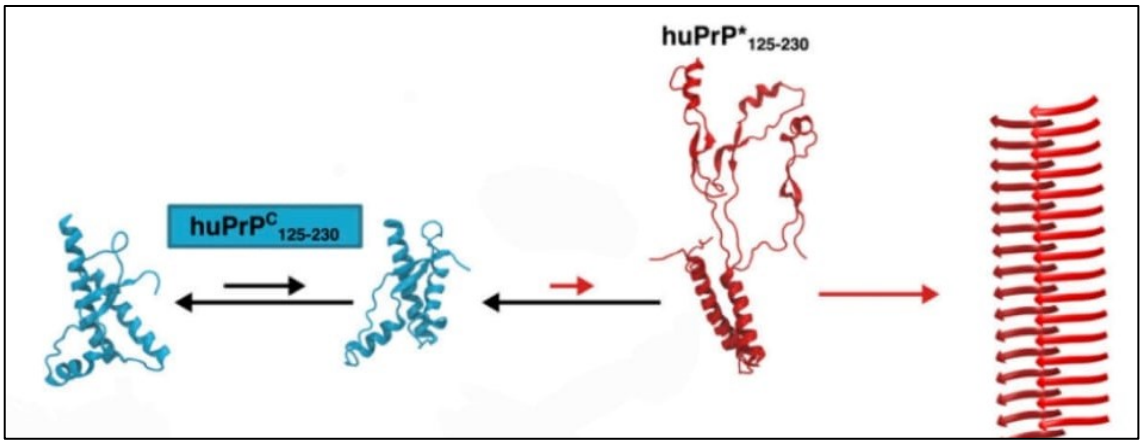

Figure 7: Process of misfolding of the cellular Human Prion Protein (in blue) into aggregates (in red at the far right of the image) and going through an intermediate named $huPrP^{*}$. This intermediate form is characterized by the detachment of the two β-strands and their attached α-helix which is helix 1(32). Extracted and simplified from "Mechanism of misfolding of the human prion protein revealed by a pathological mutation" (77)

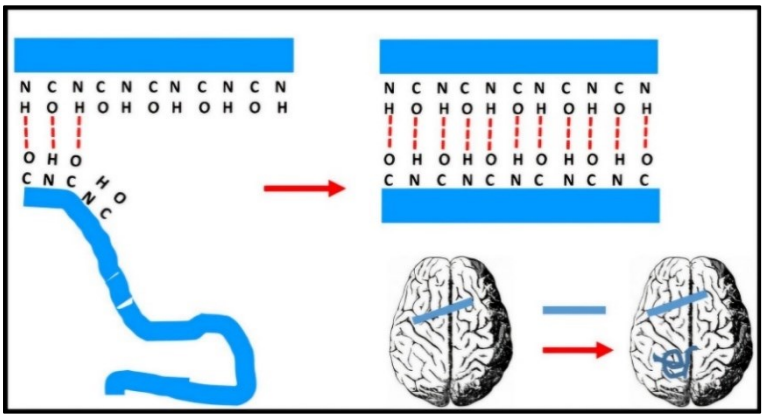

Figure 8. In this figure the edge β-strands of PrPSc are ready to form hydrogen bonds with an incoming, partially unfolded PrP^{c} polypeptide, coercing it to refold, and form fresh β-strands. This way PrP^{Sc} can propagate throughout the brain (82).

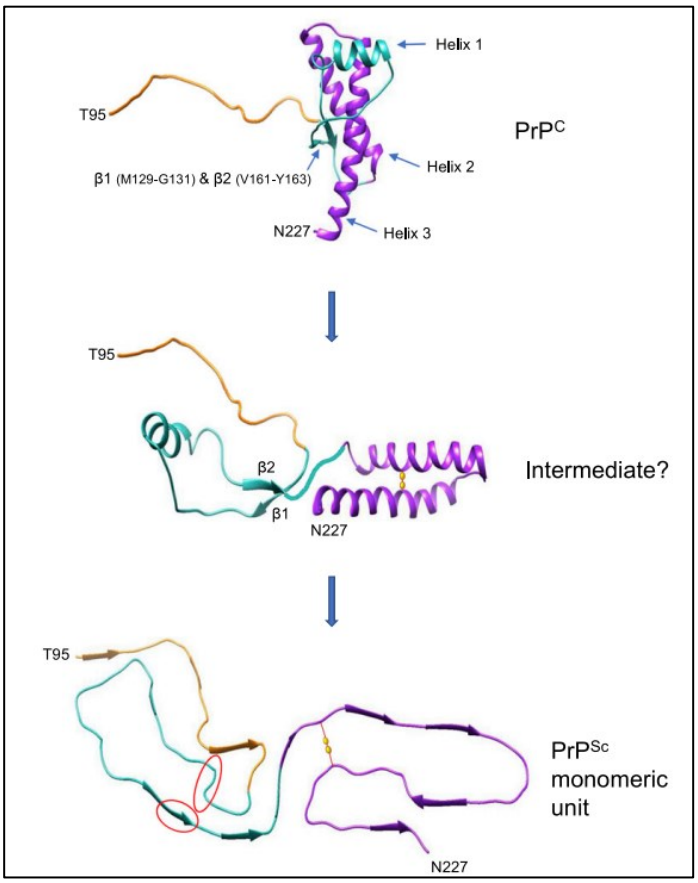

Figure 9: Transition from PrP^{c} (Top) to PrP^{Sc} (Bottom) here depicted as a monomer. The new positions of the β-sheet hydrogen bonds, as they turn intermolecular in PrPSc, is circled in red (78)

- <u>Mechanisms of replication</u>

Once the process by which the cellular prion protein misfolds and assumes a specific configuration has been elucidated, it becomes necessary to demonstrate how a protein can propagate and induce disease without other agents. The prevailing view among researchers is that PrP^{Sc}, once formed or introduced into a new host, will encounter its healthy counterpart (PrP^{c}) and refold it into a new PrP^{Sc} alternative isoform (80). This first step then initiates an exponential propagation of PrP^{Sc} isoforms that will spread throughout the body since, as we have previously stated, PrP^{c} is ubiquitously expressed throughout the body (23,80). Many experiments have confirmed this hypothesis, the most pertinent being that $Prnp^{0/0}$ mice are resistant to prion disease, meaning the endogenous PrP^{c} is necessary for developing pathology (80). As previously stated, once formed, PrPSc tends to form aggregates, also called amyloid fibrils. These fibrils are extracellular protein aggregates with particular characteristics, including a high β-sheet content,

insolubility, and resistance to degradation (81,82). The amyloid structure formed upon aggregation has two relevant structures for propagation. The first one is the Parallel In-Register Intermolecular β-Sheet (PIRIBS) structure, where different PrP^{Sc} monomers stack up on top of each other (82). Although historically associated with PrP^{Sc} strains having little to no infectivity, this structure has been associated with a hamster-adapted, fully infectious scrapie prion strain named 263K (78,83). In 2021, Kraus et al. were able to determine for the first time the near-atomic structure of the 263K PrP^{Sc} strain, showing a PIRIBS-based architecture with N-linked glycans and GPI-anchor projecting outwards from the fibril core (78). These projections are then able to distort the cellular membrane, which is in accordance with observations made in prion-infected tissue (i.e., spongiform degeneration of the brain) (Figure 10) (78,83).

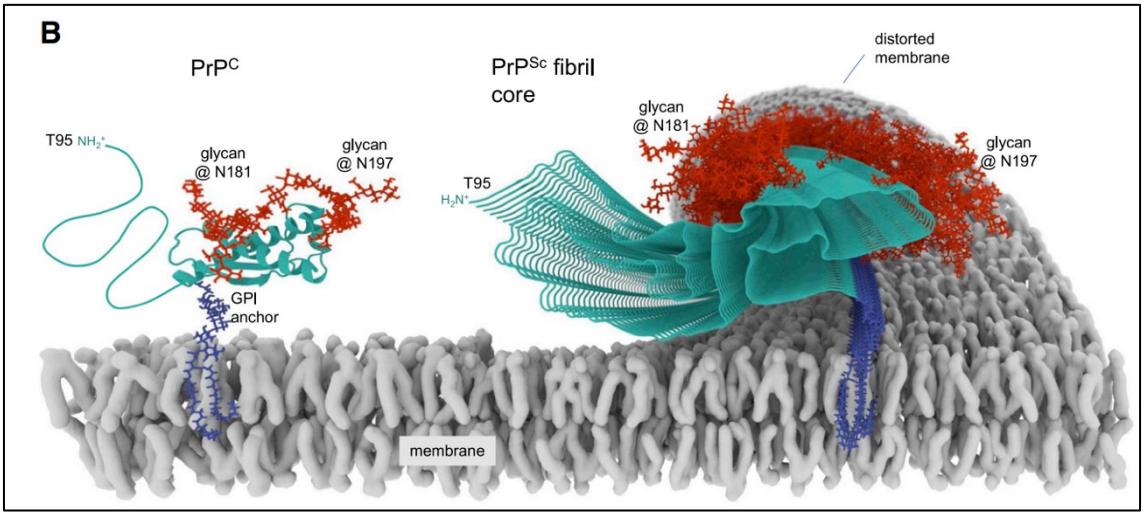

Figure 10: Left side of the picture shows Membrane-bound PrP^{c}, and right side represents the 263K prion model of the Parallel In-Register Intermolecular β-Sheet (PIRIBS) fibril core (turquoise), with hypothetical illustrations of glycans (red), the GPI anchors (blue), and the curl of the membrane (grey) is shown to follow in parallel of the twist of the GPI anchors (78).

The second relevant structure adopted by PrP^{Sc} for propagation is a four-rung β-solenoid (4RβS) structure. In this sequence, PrP^{Sc} coils around itself, with β-strands positioned on top of other β-strands of different sequences. Consequently, the stacking of these strands is not in register. An example of a prion strain adopting this type of amyloid fibril structure is the Rocky Mountain Laboratory (RML), a murine scrapie strain. RML has two different presentations, the wild-type (wtRML) and the anchorless strain (aRML), which is the subject of much more extensive study, lacks GPI-anchors and is severely

depleted in N-linked glycans (84). Nonetheless, despite the presented differences between wtRML and aRML, the amyloid fibril core is thought to be roughly the same (84,85).

Figure 11 compares 263K and RML strains and shows their amyloid structures' differences (85). Furthermore, it is crucial to highlight these distinctions, as they may offer insights into the existing obstacles to transmission between certain species (78,85).

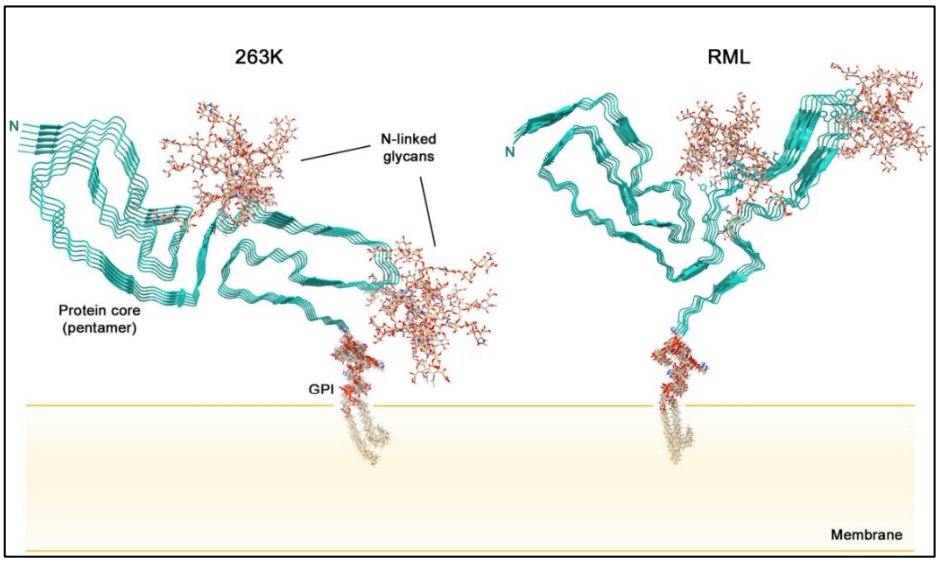

Figure 11: Cross-sectional simplified depictions of membrane-bound 263K and RML prions. The RML structure shown was assembled using the aRML PDB coordinates (PDB: 7TD6) because the wtRML were not yet publicly available (85).

I.3. Prion diseases

Prion diseases are infectious neurodegenerative diseases that are invariably lethal and affect humans and domestic and wild animal species (86,87). As previously stated, these diseases are characterized by the misfolding of the endogenous PrP^{c} into PrP^{Sc} that will accumulate in amyloid fibrils and cause cell necrosis (85). Prion diseases are also known as Transmissible Spongiform Encephalopathies (TSE) due to their infectious nature and the spongiform aspect they induce, which results in cell death in the brain. These diseases can be divided into three categories: sporadic, inherited and acquired (88). This chapter will describe the principal types of prion disease and their clinical expression. To facilitate the comprehension of the reader, Figure 12 depicts the main functional areas of the brain with their associated functions and anatomical location (89).

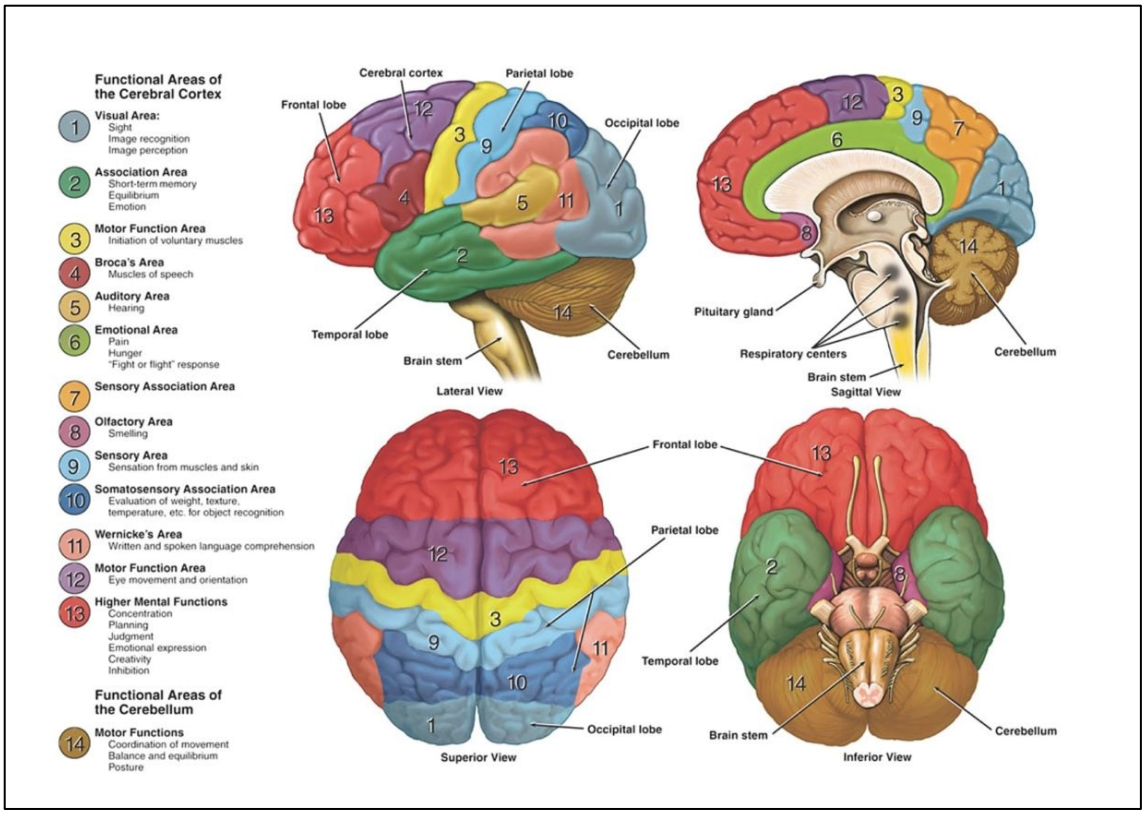

Figure 12: Image depicting the main functional areas of the cerebral cortex and the cerebellum (89).

- <u>Human prion diseases</u>

Sporadic prion diseases

Human prion diseases are mostly sporadic/idiopathic (85%), meaning their exact aetiology is unknown, but most often attributed to spontaneous PrP^c misfolding or somatic mutation of the PRNP gene (88,90). Within this classification, the most common is sporadic Creutzfeldt-Jacob Disease (sCJD), followed by sporadic Fatal Insomnia (sFI), also called the thalamic form of sCJD. Sporadic fatal insomnia is the rarest type of sporadic prion disease. It is also very similar in terms of clinical features to Fatal Familial Insomnia (FFI), which we will mention in the next paragraph (91,92).

Sporadic Creutzfeldt-Jacob Disease has a global yearly incidence of about two cases per million individuals, classifying it as a rare disease (93). sCJD covers different presentations in which the symptoms observed and the prognostic of progression to dementia, then death, will vary. These differences in phenotype are mainly divided into two main groups: the cognitive subtypes and the ataxic subtypes (91). These subtypes can be further classified into six specific categories (MM1, MV1, VV1, MM2, MV2 and

VV2) based on a specific polymorphism of the PRNP gene at codon 129, in which a methionine (M) is replaced by valine (V), and on the molecular weight of the unglycosylated PrP^{Sc} (type 1 or 2) (94). The most prevalent form of sCJD is the cognitive form (MM1 and MV1), which accounts for approximately 40% of cases (94). These present first with rapid cognitive decline and myoclonus, ataxia, pyramidal and extrapyramidal symptoms, characterized by a fast progression to akinetic mutism and death (median: 3 to 4 months survival after the onset of symptoms) (93–95). The onset of clinical symptoms is in accordance with the first affected areas of the brain by this subtype, namely the striatum (Figure 13) (holding an important role in procedural learning and memory) and the parietal cortex (Figure 11) (important in somatosensory information, having specific areas relevant for cognitive functions such as reading and mathematical thinking) (96–98).

The second most common is the VV2 or ataxic subtype, representing around 15% of cases (94). In this phenotype, patients present initially with ataxic symptoms, almost invariably accompanied by gait difficulties and frequently accompanied by associated vertigo. Subsequently, patients experience memory loss and rapid cognitive decline, accompanied by signs and symptoms similar to those observed in the cognitive subtype of sCJD (91,99). This difference in the clinical onset of the disease is explained by the part of the central nervous system that is affected first, in this case, the cerebellum, which is responsible for movement coordination, among others (Figure 12). This phenotype is also characterized by a longer duration of disease (median of 6 months) (93,95).

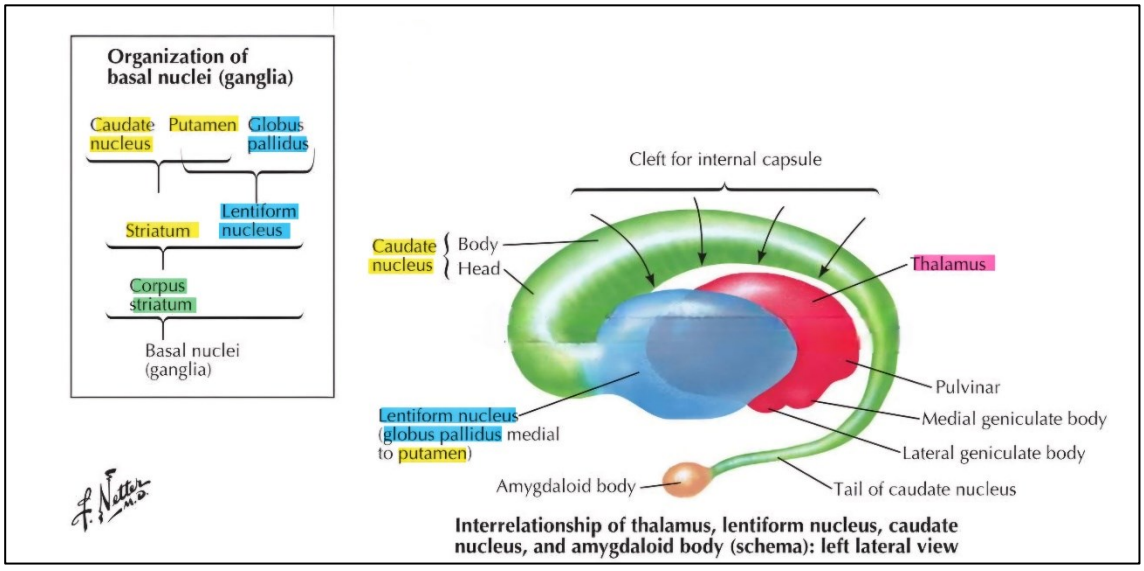

Figure 13: Organization and depiction of the brain's basal nuclei. Highlights for the Striatum and it's composing structures (yellow), the Lentiform Nucleus and it's composing structures (blue), and the Thalamus (Pink). Extracted and adapted from "The Atlas of Human Anatomy" (214)

Inherited Prion diseases

Genetic or inherited prion diseases account for 10 to 15% of all cases of prion disease (19,100). To the present day, more than 30 disease-causing mutations located in the open reading frame of PRNP have been characterized, mainly divided into three categories: single point mutations, STOP codon mutations, and insertions or deletions of nucleotides into the coding DNA of the octapeptide repeat regions (19,88,101). These mutations are then separated into a set of clinical and pathological features, diving them into three known diseases: genetic CJD (gCJD), Gerstmann-Sträussler-Scheinker Syndrome (GSS), and Fatal Familial Insomnia (FFI) (19). Furthermore, experiments reveal that these forms of spongiform encephalopathy are transmissible, granting these diseases a unique characteristic of being inherited and transmissible (88). In the case of gCJD, the symptoms are similar to those found in the cognitive presentation of sCJD. The differences are mainly in the age of symptom onset, which occurs at an earlier age (30 to 70 years old for gCJD, compared to 55 to 75 years old for sCJD), and the person has likely a family history or a positive genetic test for a mutation associated with gCJD (19,102).

GSS was first described in an Austrian Family in 1936 by Gerstmann, Sträussler, and Scheinker (103). This form of inherited prion disease develops between the ages of 40 and 60 and has an average life expectancy of 5 years after the onset of symptoms (104). These typically manifest as an abnormal gait and abnormal eye movements, such as nystagmus, in relation to early cerebellar dysfunction (103,105). As the disease progresses through the various parts of the brain, other symptoms may emerge, including memory loss and rapid cognitive decline (106). This particular disease shows a broader span of phenotypes than other inherited prion diseases, dividing itself into kindreds, making it especially difficult to diagnose (102,107).

Fatal Familial Insomnia was first named by Lugaresi et al. (1986) (108). Before this, FFI was described as a severe thalamic atrophy (92). The latter relates to the area of the brain that is most prominently affected by the disease − the thalamus – (Figures 12 and 13), which can be divided into five major functional components: dealing with arousal and pain regulation; regulating sensory domains except for olfaction; controlling motor language; cognitive function; and regulation of mood and motivation (109). The typical age of onset for FFI is between 51 and 60 years old, with a survival time of slightly over a year, on average, approximately 16 months. The symptoms of FFI can be divided

into three main groups: sleep disturbances, autonomic symptoms linked to sympathetic nervous system hyperactivity (which include hypertension, tachycardia, excessive sweating, and elevated body temperature, among others), and classic prion disease symptoms (92). Furthermore, two phenotypes are described, each belonging to a genotype. These are the 129MM and the 129MV genotypes. The first genotype is homozygous for methionine in the codon 129 and is associated with a shorter lifespan than the heterozygous one (129MV). Moreover, 129MM appears to be associated with more severe symptoms of insomnia, myoclonus, special disorientation, hallucinations, and autonomic dysfunction (92,102). While the heterozygous genotype appears to be clinically closer to classic CJD symptoms, including worsening equilibrium and ataxia, FFI's genotypes are associated with an early onset of sleep disruption, characterized by progressive sleep time and quality loss. The prolonged severe insomnia is associated with the overactivation of motor functions and the sympathetic nervous system, which will result in not only autonomic dysfunction but also a behaviour/symptom called *agrypnia excitata* with oneiric stupor. The latter is identified as an extended episode of dream reenactment, often consisting of movements executed in daily activities, that can be remembered upon "awakening". *Agrypnia excitata* with oneiric stupor in FFI is usually defined by longer and almost continuous episodes compared to other REM sleep disorders (92,110). Finally, as the disease progresses, classic TSE symptoms begin to appear, in particular cognitive decline, which in this form of prion disease is characterized by alterations in the level of vigilance leading to a progressive dream-like state (92).

Acquired Prion diseases

Acquired prion diseases are transmitted most often by the ingestion of contaminated meat. The first acquired form of TSE described in humans was Kuru disease, which derived from cannibalism practiced in indigenous tribes, where the contents of the brain of deceased people were consumed in rituals (111). More Recently, a zoonotic TSE surfaced when cases of CJD appeared to have an increased incidence. It was later discovered that these cases arose from the consumption of bovine meat from cattle affected with Bovine spongiform encephalopathy. This variation of CJD is now referred to as variant CJD (vCJD) (112–114). The last type of acquired prion disease is iatrogenic CJD, which happened following blood transfusions and use of contaminated material with PrP^{Sc} (115–118).

I.4. Neurodegenerative Proteinopathies

Many neurodegenerative diseases are associated with proteins endogenous to the human body, that misfold and generate β-sheet rich isoforms that tend to aggregate and cause disease (119,120). This mechanism of aggregation and self-templating has been described increasingly as being a "prion-like" mechanism in reason of the similarities exhibited with prions (121–124). Of the diseases described as to having this behaviour, Parkinson's and Alzheimer's diseases are the most studied to this day (119,125).

I.5. The Gut-Brain axis

In recent years researchers have increasingly studied a phenomenon called the gut-brain axis (126). This communication pathway between the CNS and the gut with its associated functional tissues and microbiome, is a two-way crosstalk where not only the brain triggers the gut, but the contrary also happens. Increasing evidence arises proving that gastrointestinal health influences neuronal, and overall mental health (127). Moreover, proof has been shown that bacterial byproducts are recognized by the central nervous system and possess the ability to modulate behaviour in mice (128). These information push further researchers to study the gut-brain axis in the development of neurodegenerative disorders in order to better understand them.

II. Methods

II.1. Aim

The present review aims to collate the extant knowledge on the associations between the gut and prion or "prion-like" diseases. To facilitate the screening process, research questions were designed to guide reviewers through the execution of this review. Moreover, although this work does not fully meet the criteria for classification as a systematic review, it is nevertheless intended to be as systematic and reproducible as possible.

Q1: Is there a relationship between the gut and the future development of prion disease or other neurodegenerative proteinopathies?

Q2: Do prions and other misfolded proteins take the same paths from the gut to the central nervous system?

Q3: Is there any evidence supporting a connection between classic prion diseases and neurodegenerative proteinopathies concerning the gut?

II.2. Materials and research methodology

The articles for this review were collected by one researcher (M.T.), who defined a search keyword algorithm intended to encompass the most relevant articles for the review. The search algorithm employed was as follows: "((prions) AND (microbiome OR gut))". To ensure comprehensive coverage of the available literature, six databases were consulted, with the same search algorithm employed in each. These were PubMed, Scopus, Embase, Cochrane, B-On and Web of Science. The search algorithm was modified slightly between databases to align with their respective parameters. No time limit was settled, nor any language was excluded while executing this research. To mitigate the risk of bias and ensure the replicability of the process, a second reviewer (R.C.) undertook the processing of the search in all the databases, thereby ensuring no discrepancies between the reviewers. The final search was conducted across all databases on the 1st of December 2023. Three individuals screened the titles, abstracts, or full references from the search results to assess their eligibility for inclusion in the review.

The screening process was conducted using the "Rayyan" (129) online application, specifically designed to assist with the management of literature reviews.

II.3. Primary screening of articles and inclusion criteria

The initial screening of the articles was conducted on the basis of title, keywords and abstract, with duplicates being excluded. To reduce the selection bias, three reviewers engaged in the selection process (I.M., M.T. and R.C.). In this first phase, the inclusion criteria were every primary article that connected the gut and prion or "prion-like disease". The reviewers were required to select articles on neurodegenerative diseases for which a misfolded protein with the propensity to form aggregates and "self-replicate" in a prion-like manner was responsible. This definition of "prion-like disease" was used to guide the selection of articles (130). The exclusion criteria excluded articles that were focused on diagnosis and biomarkers for diagnostic tools and epidemiologic studies that would not answer the research questions. Furthermore, background articles (i.e., articles that did not involve any original experimentation) were also excluded. Nevertheless, those deemed relevant for the review were designated "on topic" to review the original articles referenced to ensure the search was as comprehensive as possible. Each of the three reviewers conducted the review process independently, with no access to the others' inclusion decisions. Once each reviewer had completed their selection process, M.T. was able to unlock the decisions made by each reviewer. This then allowed for a discussion between all three reviewers to assess cases where there were conflicts in the decision to include or exclude the article.

II.4. Secondary Screening

The secondary screening was done by one reviewer (M.T.), to classify these articles by their population and the subject they tackled in relation to our research questions. The secondary selection process involved a more comprehensive procedure whereby the reviewer conducted a more thorough examination of the abstract and the full text, ensuring that it met the inclusion criteria for this review. To facilitate the classification of the primary articles, labels were introduced to distinguish between them according to population and the theme relevant to the review (Figure 14).

Population:	Principal subject(s):
	○ Degradation of Prp^{Sc}/misfolded protein in the GI tract
○ in vitro	○ Enteric Nervous System (ENS)
○ Nematodes	○ Pathogenesis and location of Prp^{Sc}/misfolded protein after oral
○ Animals	or parenteral challenge
○ Humans	○ Prion/misfolded protein uptake from the gut
	○ Prion susceptibility
	○ Gut Associated Lymphoid Tissue (GALT) association

Figure 14 : Article labelling

III. Results

III.1. Description of the results

Of the 514 articles initially identified, 385 were excluded for the following reasons: they were review articles, they were outside the scope of the review (off-topic), they were of the wrong publication type (in this case, a protocol) or they had the wrong outcome. The results are presented in a flow-chart PRISMA diagram (131) (Figure 14). At the second screening stage, a further 23 of the 129 references were excluded, leaving a total of 102 articles included for the final review. Of these, 85 were about prion diseases, four were about Alzheimer's disease (AD), and 13 were about Parkinson's disease (PD). Out of the 102 references, 68% were studies carried out in animals, more precisely in mammals, 20% were *in vitro* studies, while human tissue samples (including biopsies) constituted 5%. For further details, please refer to Table 1.

Table 1: Summary of the references kept for the final review separated by disease and population

POPULATION	Parkinson's disease	Alzheimer's Disease	Prion disease
Animal trials	8	2	59
in vitro	1	0	20
Animal trials and *in vitro*	0	2	2
Animal trials and nematodes	1	0	1
Nematodes	1	0	0
Human tissues	2	0	3
TOTAL	13	4	85

Three studies included nematodes (roundworms), two of which were conducted with *Caenorhabditis elegans* (132) and one with *Trichuris muris* (133). The earliest article in this review was published in 1989, but the majority (99/102) were published after the year 2000.

III.2. Prion/misfolded protein's uptake from the gut

In this review, a total of 72 references mention the prions or misfolded protein's uptake from the gut. Of these, 63 focus on prion protein, three on Alzheimer's Disease related proteins, and nine on α-Synuclein (PD).

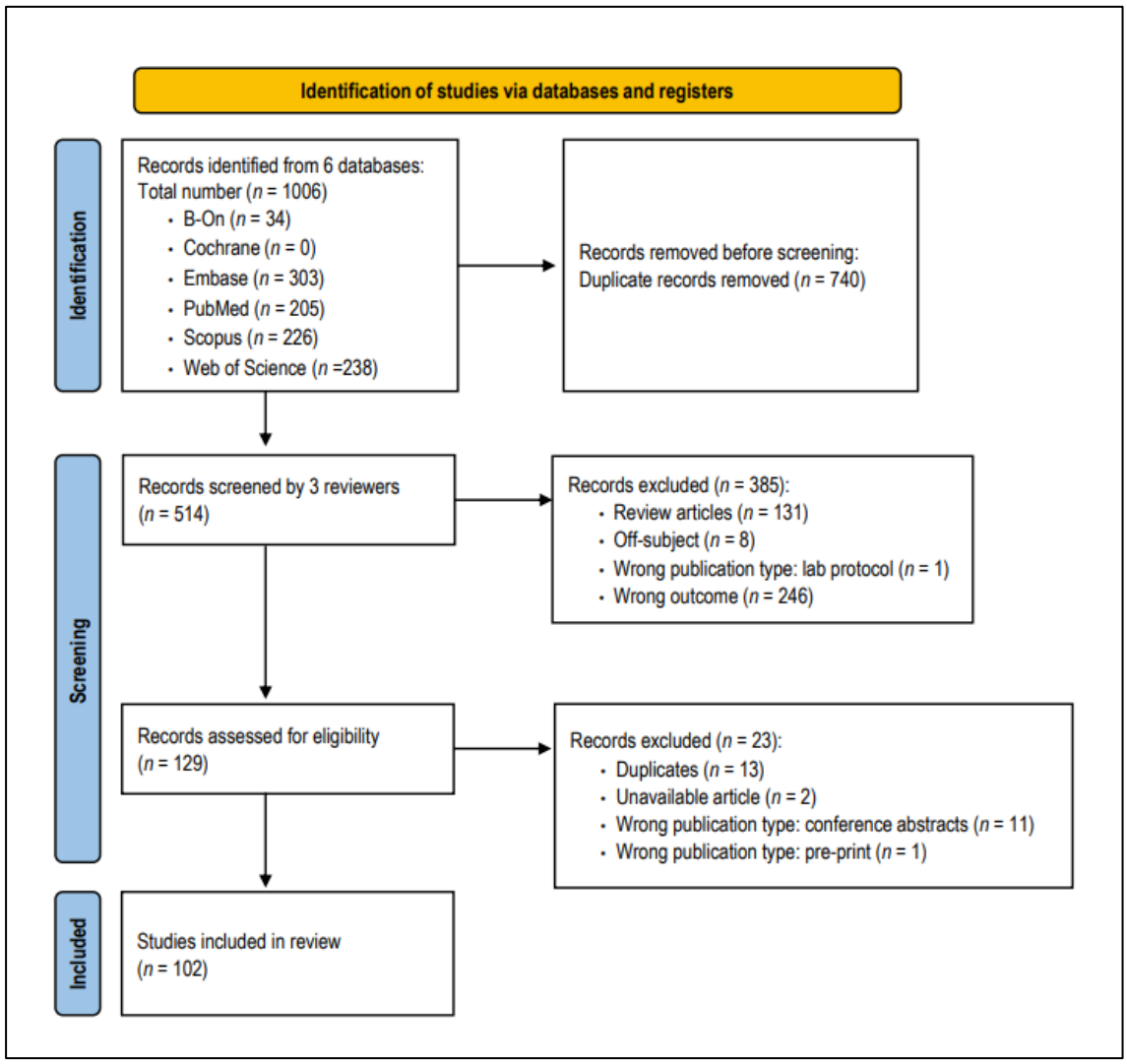

Figure 15: PRISMA Flow diagram from: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. (131)

Prions

In the case of prion diseases, it is known that, when they are acquired, they are generally transmitted orally. This is the case for example in Bovine Spongiform encephalopathy, vCJD and Kuru (134,135). In this section, will be presented the results of the review pertaining to PrP^{Sc} uptake from the gut lumen to the CNS, passing through various structures.

Forty-eight references mention the importance of the Gut-Associated Lymphoid Tissue (GALT) in the uptake of misfolded prion proteins, but also as an early replication site for PrP^{Sc} before neuroinvasion. Donaldson et al. (2015), stated that the small intestine's GALT plays a major role in PrP^{Sc} invasion, particularly when compared to the large intestine, which seems to bear little to no significant effect on pathogenesis (136). As early as the year 2000, Beekes & McBride observed scrapie infect mice after oral challenge and defined the GALT -particularly the ileal GALT- as an important locus for PrP^{Sc} intake and propagation, with follicular DC being the most concentrated in PrP^{Sc}. Within the GALT, Peyer's Patches (Figure 5) play a decisive role in PrP^{Sc} accumulation (137), and within these structures Dendritic Cells are another important stakeholder in the uptake of misfolded prions. Huang et al. (135) demonstrated that DCs can "take up and transport PrP^{Sc} from the gut lumen through the lymphatics to lymphoid tissue". This was further proven by Raymond et al. (136) who showed that, when $CD11c^{+}$ DCs were depleted in the GALT and spleen before oral challenge in mice, early PrP^{Sc} accumulation in these tissues was blocked, therefore susceptibility to disease decreased and neuroinvasion by PrP^{Sc} was impaired. Following this study, Bradford et al. (137) demonstrated that chemokine receptor expressing DCs (CXCR5) propagate prions towards follicular DCs after oral challenge, by inhibiting specifically their expression in orally infected mice, who showed a reduced early accumulation of prions upon follicular DCs in the Peyer's patches and spleen, significantly reducing disease susceptibility.

DCs are also seen, as shown in Figure 15, near Microfold cells (M cells) beneath the follicle-associated epithelium (FAE) (138). Van Keulen et al.(138), describes M-cells as a potential pathway from the lumen to the GALT in sheep highly susceptible to scrapie infection. In addition to this, Miyazawa et al. (139) were able to prove that murine-adapted BSE were transported by M cells in an *in vitro* model of bovine intestinal epithelial cell line, this model had a 30-fold increase in the uptake of prions when compared to non-differentiated cells. Donaldson et al. (140,141), further proves involvement of M cells in prion uptake by orally challenging mice with ME7 scrapie strain. In 2012, his team showed that when mice were depleted of M cells, early prion accumulation in the follicular DCs of PPs is blocked. In 2017, the contrary experiment was carried out, showing that mice over expressing M cells had a "10-fold higher infectious titre of prions", and experienced shorter survival times (143). In a different type of experiment, Foster & MacPherson (142) tested ileal and caecal PP in their uptake of PrP^{Sc} in orally challenged murine. In this study they found that PrP^{Sc} was bound to M

cells and enterocytes of the follicle-associated epithelium in ileal PP within 24h of inoculation. Furthermore, within 48 h after ingestion PrP^{Sc} was bound to M cells and enterocytes of the follicle-associated epithelium in caecal PP, it was also found that the greatest accumulation of PrP^{Sc} happens in the region of the caecum over the 48-hour period studied. It was suggested by the authors that this anatomical region might act as a retention pouch for PrP^{Sc} upon oral infection. In 2015, Nagasawa et al. showed that M cells and goblet cells (Figure 5) synthesize aldolase A which in turn binds to prion protein, leading the investigators to think that prions can be taken up by M cells in a PrP-Aldolase A complex. However, in 2011 and 2012 *in vivo* studies made in sheep by Åkesson et al. demonstrated that the follicle associated epithelium of PP was not involved in PrP^{Sc} uptake, whereas the absorptive epithelium had evidence of PrP^{Sc} uptake (Figure 16). However, in 2012 Åkesson et al., further examined the phenotype of the cells transporting exogen PrP and found that DC were not co-localized with the studied recombinant-PrP, instead it was associated with macrophages or immature DCs. The entry of PrP^{Sc} into the GALT by the absorptive epithelium, was further supported by Jeffrey et al. (143), and Dagleish et al. (144), which localized PrP^{Sc} in the lacteals and submucosal lymphatics, of sheep gut loops within 15- or 30-min post inoculation respectively, and up to 3,5 hours post-challenge (Figure 16a). Ano et al. (145), was able to make the same type of observations after orally infecting 15-day-old mice with scrapie-associated prions. However, 30 days post infection PrP^{Sc} was detectable in the follicles of PP, namely inside macrophages (Figure 16) (146,149,150). Similar to this, Okamoto et al. (148), showed that in neonatal (one day old) mice orally infected with scrapie-associated prions, PrP^{Sc} was localized in the cytoplasm of villus enterocytes 1 hour after inoculation.

Relating to the mechanism by which PrP^{Sc} is transported from the lumen to the follicular DCs, Marshall et al(149) showed that intrinsic PrP^{c} expression by enterocytes is not necessary for PrP^{Sc} uptake and early replication in Peyer's patches. This experiment was able to show that after oral challenge, the quantity of PrP^{c} inside enterocytes did not influence susceptibility to disease.

In 2006, Austbø et al. found that PrP^{c} expression levels were low in lambs' lymphoid follicles, while high concentrations were detected in the outer submucosa and muscular layer of the gut. This suggests that while the GALT may act as a reservoir for PrP^{Sc} accumulation, it is not the main site of protein conversion (153). Moreover, inside PP, PrP^{Sc} is thought to accumulate in the germinal centres which are not directly innervated, however PrP^{c} is intensively collocated with the DC present in the

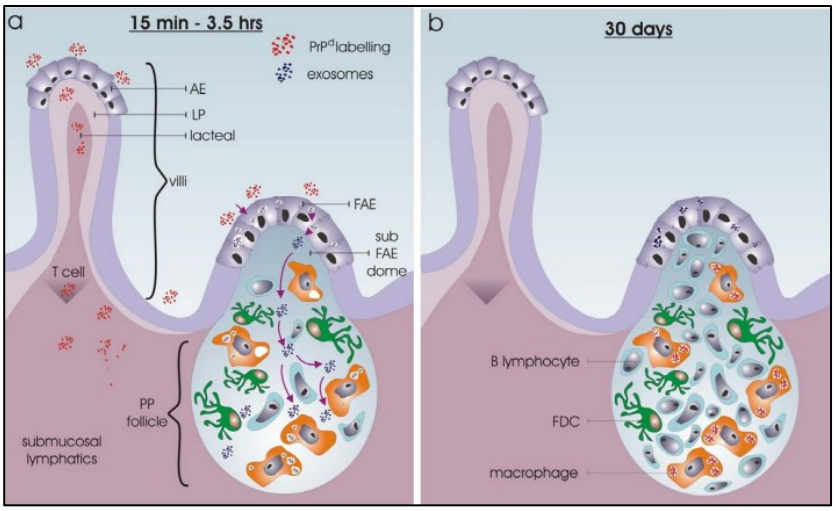

Figure 16: a) PrP^{Sc} localization within 15 minutes to 3,5 hours after oral challenge in ileal PP **b)** PrP^{Sc} localization 30 days after oral challenge in ileal PP. FAE: follicle-associated epithelium (149)

suprafollicular area of PPs, these dendritic cells establish contacts with follicular dendritic cells and with B cells inside germinal centres possibly carrying PrP^{Sc} in cases of infection (154). Furthermore, it was found that in mice with a reduced number of PP, oral challenge became impossible, strengthening the hypothesis of PP being necessary for prion oral infection (154,155). Furthermore, in same study Prinz et al. (152) was able to determine that although the number of PP influences greatly oral susceptibility to scrapie-associated prion disease in mice, the number of lymphocytes associated to them do not disturb prion intake.

Glaysher et al. (2007) (156) further substantiate the significance of GALT for neuroinvasion by Scrapies in a murine experiment. In this experiment, mice that were knockouts for the GALT and follicular DC were found to be resistant to oral infection with Scrapies. In the same study, when follicular DC were reintroduced without the presence of PP and mesenteric lymph nodes, or even just in the absence of PP, mice were still refractory to oral challenge. However, upon the introduction of isolated lymphoid follicles within follicular DC (in the absence of PP), mice exhibited comparable symptoms to those observed in the negative control mice (156). In a recent study, Balcan et al.(154), observed PrP^{c} transit and localization in mice under endoplasmic reticulum stress versus basal conditions. In mice with induced endoplasmic reticulum stress, the levels of PrP were significantly increased in enterocytes and lacteals of PP (Figure 16), whereas they were decreased in M cells. This study suggests that in basal conditions, PrP

uptake from the gut lumen occurs primarily through the M cell-Peyer's patch-mesenteric lymph node axis, with an alternative pathway via the enterocyte-lacteal-mesenteric lymph node axis. However, in the context of endoplasmic reticulum stress, the enterocyte-lacteal-mesenteric lymph node appears to be the sole axis for PrP transmission.

Once PrP^{Sc} has entered the GALT, it must still cross the blood-brain barrier in order to initiate neuroinvasion. To this issue eight references pertain to the role the Enteric Nervous System (ENS) plays in this regard. In 2008, Albanese et al. found that mice expressed PrP^{c} inside enteric glial cells, these cells represent an integral part of the ENS and significantly outnumber neurons in that area. Studies have suggested that enteric glial cells exert essential roles in supporting the survival and functions of the ENS neurons (158). This localization of PrP^{c} is seen to be a potential site for neuroinvasion by PrP^{Sc}, according to (156).

Chiocchetti et al. (157) concluded, after observing the GALT from sheep ileum (distal part of the small intestine), that enteric and sympathetic neurons innervate the lymphoid follicles of Peyer's patches, therefore constituting a possible pathway for prion neuroinvasion. However, McGovern et al. (158), found that nervous cells and PrP^{Sc} are only rarely located together inside PPs, suggesting that even if accumulation happens in the germinal sites of PP (Figure 5) neuroinvasion happens within other structures. On the other hand, in the same study it was observed that "sheep between 2 and 4 months of age had significantly more nerve fibres within follicles than older groups", which could probably explain why young sheep are more susceptible to prion disease when compared to older animals (161). In addition to this, St. Rose et al. (159) observed a strong negative correlation between the concentration of PP and number of associated lymphoid follicles, with the age of Cheviot sheep. The reduction in PP and associated lymphoid follicles is correlated with an increase in the age of sheep.

In 2010, Dagleish et al. (147) arrived at the conclusion that Scrapie-causing PrP^{Sc} is more proteinase resistant than BSE-causing PrP^{Sc}, by inoculating sheep and observing gut loops 15 min up to 3,5 hours after direct inoculation into the loops. In 2000, 2002, and 2003 Heggebø et al., published a series of studies made in lambs and sheep. The first study stated that the distribution of prion protein -no discrimination was made between PrP^{c} and PrP^{Sc} - within the ileal PP of lambs (5 months old) changes between a scrapie-free individual and an infected one however, the distribution of prion protein was similar between naturally infected lambs and those who were inoculated by a single dose scrapie-infected brain given orally (Heggebø et al., 2000). The second study by Heggebø et al.

(161), was focused on the distribution of PrP^{Sc} in the GALT of 20 to 24 months old sheep with late pre-clinical signs of scrapie and early clinical signs. This experiment first showed that between the early clinical stages of diseases and the pre-clinical stage there is a difference in the colonization by PrP^{Sc}, with the individuals being in the pre-clinical stage only showing limited lesions in the brain, whereas in sheep that had begun to show symptoms PrP^{Sc} was widely spread throughout the CNS. However, despite the differences observed in the CNS, concentrations of PrP^{Sc} in the lymphoreticular system and peripheral nervous system were comparable between pre-clinical and clinical stages of disease. Furthermore, a large accumulation of PrP^{Sc} was found in lymphoid nodules of the alimentary tract, and other lymphoid tissues were also affected including extra-nodular sites of lymphoid tissues, such as the marginal zone of the spleen. The third article published by Heggebø et al. (162), was made from the same experiment as the previous one, only this time, the functional region targeted was the ENS. This study found that PrP^{Sc} was more prominent in the ENS when the disease-associated protein had already invaded the surrounding lymphoid tissue. Furthermore, this experiment revealed that lymphoid follicles are highly innervated, which is confirmed by other authors (161). Keulen et al. (141), in a study describing early and late pathogenesis of natural scrapie infection in sheep, defines the first stage of infection as the colonization of the GALT by PrP^{Sc}, the second as the access to the efferent lymph and subsequently the blood stream leading to the "dissemination of the scrapie agent to non-GALT-associated lymphoid tissues", and the final stage being neuroinvasion which in this study starts in the ENS supporting the findings of other studies (153,154,165,166). In 2014, González et al. (167) observe the pathogenesis of PrP^{Sc} in sheep using two inoculation methods, oral and conjunctival (conjunctival tissue of the eyes) challenge. Despite the difference in route of infection both populations showed identical tissue progression, the earliest detection of PrP^{Sc} was observed in gut- and pharynx-associated lymphoreticular tissues. After this, the brain and other lymphoreticular tissues became simultaneously PrP^{Sc} positive, and lastly the spinal cord and peripheral nervous tissues of the enteric, parasympathetic, and sympathetic systems became affected. In contrast, Kimberlin et al. (1989), found evidence of PrP^{Sc} replication in the spinal cord before finding it in the brain (168). Furthermore, irrespective of the inoculation method sheep showed identical symptoms, which started 6 and 8 months after infection for oral and conjunctival challenge, respectively. The commonalities observed between these two routes suggest "early dissemination of the infectious agent in the bloodstream and a common neuroinvasion

pathway," (167) supporting the haematogenous route of infection to the brain previously described by Van Keulen et al. in 2002 (138), although in this case the ENS was not the first nervous tissue to be affected.

Similarly to Scrapie in sheep, CWD in deer population appears to have the same pathogenesis upon oral infection, as depicted by Sigurdson et al. (169).

In contrast with the findings in ovine population, Defaweux et al. (167) studied the amount of nerve fibres in Ileal PP of bovine cattle, and found that the older the animals were, the more nerve fibres were present in the germinal centres of PP in the ileum of calves, specifically near follicular DCs. These observations lead to see the innervation of the germinal centres to be an "age-dependent dynamic process" (170), possibly influencing the path of neuroinvasion in bovine cattle and their susceptibility to BSE. In more recent studies (171,172), four- to six-week-old calves were orally challenged with BSE-causing PrP^{Sc} and their peripheral nerves and ganglia, as well as central nervous tissues were sampled (from 1week to 8 months post oral challenge). The results of this study showed that, upon inoculation of a high dose of PrP^{Sc}, the calves showed infectivity in ileal PPs as early as two-month post-inoculation, and, as early as 8 months after infection in the spinal cord and parasympathetic nodal ganglion located close to the brain. However, it has been shown that the incubation period is dose-dependent, therefore the early observation of PrP^{Sc} in central nervous system is influenced by the dose given to the calves (100g compared to the 1mg dose that has been shown to be sufficient to cause an infection in cattle). Studies done with older calves aged 4 to 6 months (173,174), with the same inoculating dose as Ackermann et al. (168), searched PrP^{Sc} in the small intestine from 1 month to 44 months post-infection. Infectivity in these animals was detected not only in the ileum but also in the ileocecal junction and in the jejunum from 4 months post-infection up until 44 months. The same observations were made by Okada et al. (147), this time with calves that were 3 to 4, and 9 to 11 months old and sacrificed at 20, 30 and 46 months post oral challenge. Furthermore, no PrP^{Sc} was observed in the myenteric and submucosal plexuses of the ENS of the small intestine or any other tissues. No observations were made on the age differences between the calves. However**,** higher transmission rates and shorter incubation periods were obtained from ileal samples proving their higher infectivity compared to other small intestine regions (174,175). Fast et al. (170), also reported in their follow-up study that the amounts of infectivity observed at 4 months post-infection are comparable to levels at later stages of disease.

In BSE several articles point to the importance of the 37-kDa laminin receptor (37LRP or RPSA)(176), in prion uptake from the gut. In vitro, BSE is endocytosed by Human enterocytes via the 37 kDa/67 kDa Laminin Receptor. A study done in 2005, showed that PrP^{Sc} was internalized via endocytosis within minutes of infection. The endocytosis of the infectious BSE-associated PrP^{Sc} was reduced when the 37 kDa/67 kDa laminin receptor (LRP/LR) was inhibited. This receptor, which is apically expressed in Caco-2/TC7 cells, seems to be the entry point for the BSE-associated PrP^{Sc}. The results from this study, therefore, underscore a potential role of enterocytes in the absorption of BSE-associated PrP^{Sc} during oral infection through specific 37 kDa/67 kDa laminin receptor-dependent endocytosis (177). Moreover, porcine species are immune to oral transmission by BSE-associated PrP^{Sc} which might be explained by the differences between aa sequencing between pigs and ruminant mammals (178).

A study carried out *in vitro* with cell cultures of different animals suggests possible oral transmissibility of CWD and sheep scrapie to humans and confirms the oral transmissibility of BSE to humans. However, CWD does not seem to be transmissible to cattle, pigs, and sheep. Furthermore, sheep scrapie might have caused BSE but may not cause TSE in cervids and pigs, and BSE may not be transmissible to cervids. Data from this experiment however only takes the enterocyte model system into account to model prion infections (179).

In a study named "Protease-Resistant Human Prion Protein and Ferritin Are Cotransported across Caco-2 Epithelial Cells: Implications for Species Barrier in Prion Uptake from the Intestine", it is suggested that PrP^{Sc}-associated proteins, in particular in complexes with ferritin-largely conservated between species-, may facilitate PrP^{Sc} uptake in the intestine from distant species, leading to a carrier state in humans (180).

In 2015, Holznagel et al. infected orally Cynomolgus monkeys with brain of cows with bovine spongiform encephalopathy. This experiment was carried out in order to better understand the human vCJD model by using a simian subject. The results showed that BSE-associated prions were preferentially transported from the gut to the CNS along afferent sensory nerve fibres and initially entered the simian CNS at lumbar spinal cord levels. In asymptomatic animals, we found BSE in 50% and 12% of gut- and tonsil-derived samples, respectively. This study also revealed that tonsils remained negative for PrP^{Sc} for an extended period of the incubation time, mainly becoming positive during clinical phase of the disease. If neuroinvasion happens in a similar way in human

population, this suggests that tonsillectomy testing drastically underappreciates the number of silent carriers in humans (181).

Parkinson's disease

There is an increasing amount of evidence pointing to PD having two different types of disease onset being the "gut first" and "brain first" phenotypes (182). The references herein produced describe the "Gut first", phenotype of Parkinson's disease. Two of these studies have demonstrated a potential new pathway that would lead misfolded α-Synuclein from the gut to the central nervous system (183,184). Chandra et al. were able to observe that, enteroendocrine cells by their close proximity with both the gut lumen and the ENS are good candidates for α-Synuclein uptake from the gut to the CNS (184). Furthermore, the same author was able to prove that α-Synuclein not only is expressed by enteroendocrine cells, but also that the pathological isoform of α-Synuclein can go from these gut mucosal cells to the interconnected nerves. Two other studies focused on the enteric nervous system and its role in the uptake of misfolded proteins to the CNS (185,186). Heng et al. (183), treated mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is extensively used to induce parkinsonism in animals. In this study, chronically MPTP- treated mice showed an increase in abnormal aggregated and nitrated α-synuclein, particularly in the myenteric plexus of the stomach. Bencsik et al. (182), described the particular case of an α-Synuclein mutation (A53T) expressed by transgenic mice. In their case the expression of misfolded protein appeared to happen simultaneously in the ENS and CNS, however it appears that the pathogenic α -synuclein is expressed in the ENS slightly before than the CNS.

One study by S. G. Chen et al. (184), used nematodes - C. *elegans* - as a model for PD and was able to feed them exogenous re-formed α-Synuclein fibrils which induced a prion-like propagation of aggregates, mimicking the pathogenesis of PD. This study also hypothesizes that heparan sulphate proteoglycans (a molecule ubiquitously expressed in mammals' tissues in cell surface and extracellular matrix) (188), mediate the uptake of α-Synuclein fibrils from the gut lumen to the ENS. The last study was performed in human duodenum biopsies from PD patients. In this experiment, Vascellari et al. (186), were able to detect pathologic α-Synuclein aggregates in the duodenum of PD patients with the aid of a rapid, ultrasensitive seed amplification assay (RT-QuICR). This tool not only proves the presence of misfolded protein in the studied area, but also the prion-like behaviour of self-propagation it has. This study, further supports the

possible gut-to-brain pathway of misfolded α-Synuclein in the "gut-first" phenotype of PD.

Alzheimer's disease

The three articles found, focus on mechanisms "prion-like" adopted by beta-amyloid (Aβ) such as with Y. Sun et al. (173) and J. Y. H. Liu et al. (174), describing the possibility of misfolded Aβ to travel from the gastrointestinal tract to the CNS of mice namely from the myenteric plexus and through the vagus nerves. These introduce the possibility that, AD might, just like PD and prion diseases have a starting point in the gastrointestinal tract. Moreover, Flach et al. (175) have found that Sup35NM fibrils (a yeast prion) are able to initiate neurodegeneration in transgenic mice expressing the human tau protein after intra cerebral inoculation, also stating the possibility that non-mammalian prions originating from the microbiome, can originate protein misfolding in their host and be at the starting point of neurodegenerative diseases such as AD.

III.3. Prion/misfolded protein susceptibility

In this review, the question of susceptibility to prion disease generated twenty-one results in prion diseases, three in PD and one in AD. However, as most articles have two or more simultaneous labels, this subject is not restricted only to this paragraph.

In 2010, Ano et al.(130) observed that in suckling mice (i.e. 15 days old) lacking maternal antibodies, the uptake of PrP^{Sc} was done at a significantly lower rate than in wild-type mice. Moreover, PrP^{Sc} uptake increased when administered with purified immunoglobulin G (IgG). This study suggests that maternal immunoglobulin and the weaning period of animals, are important risk factors for the oral susceptibility to PrP^{Sc}.

In 2019, Sánchez-Quintero et al. showed that mice infected with a natural mouse small intestine-restricted helminth pathogen *Heligmosomoides polygyrus* (a worm-like parasite) (193), subsequently infected with ME7 scrapie-prion strain demonstrated a significant increase in survival time. However, this phenomenon only happened when prion inoculation was done 8 days after helminth infection, which corresponds to the period when the adult worms re-emerge back into the gut lumen. This time stamp also corresponds to a significant increase in Mononuclear phagocyte population, such as macrophages, however how this influences PrP^{Sc} uptake from the gut lumen is yet to be determined (194).

In 2015, Martin et al. subjected mice to a nonsteroidal anti-inflammatory drug, indomethacin. This class of drugs is known to cause intestinal inflammation, therefore expected results were that of an increased susceptibility to scrapie-associated strain ME7 upon oral challenge. However, instead of increasing PrP^{c} concentration (expected in inflammation), acute administration of indomethacin decreased cellular expression of PrP^{c}. Moreover, when administered chronically indomethacin led to a "modest delay in the onset of neurological disease" (195).

III.4. Degradation of PrP^{Sc} by the gut

Another label defined in this review was the degradation of PrP^{Sc} by the gut of affected animals, 8 references in total mention this subject, all relative to prion disease.

Krüger et al. (179), describe faecal shedding of, in hamsters after oral challenge with scrapie-associated prions at 24 to 72 hours post infection. In the first 24 hours, and late pre-clinical or clinical stages, no PrP^{Sc} was found in hamsters' excrement. Furthermore, only about 5% of the initial inoculum was found in the faeces, and the majority of PrP^{Sc} was out of the alimentary tract, and the authors attribute this mainly to degradation by digestion.

IV. Discussion

Upon completion of the review, the labels were fined in an attempt to segregate the references according to the disease, the population, and the subject matter. The initial subject under consideration was the uptake of misfolded protein or prion from the gut lumen into the gut. Regarding this subject, most articles identified referred to prion diseases, with scrapie featuring the most frequently and BSE the second most frequently.

Upon oral infection, it is agreed upon that the PP play a key role in prion uptake and replication (153,155,160,161), however the exact mechanism by which PrP^{Sc} is able to cross the epithelial barrier is still discussed. References mention on the one hand the importance of the follicle-associated epithelium of PP, namely with M cells (143–145,197), but others deny this structure's implication in the uptake of the protein stating that the absorptive epithelium of PP is the responsible structure for PrP^{Sc} uptake (149,152). Nonetheless, a recent study hypothesized that endoplasmic reticulum stress influences the pathway used by PrP^{Sc} to enter the GALT and initiate replication. This study proposes that both pathways previously mentioned exist, however if the endoplasmic reticulum exists in a stress inducing environment, then the absorptive epithelium becomes fully responsible for the uptake (157). These results show that, even if a significant number of experiments have been carried out through the years, it is still difficult to this day to define the exact point, or points of entry borrowed by PrP^{Sc}. Furthermore, the last study mentioned made its hypothesis while observing cellular prion protein and not its disease-associated isoform, limiting therefore the conclusions that can be taken from it. Nonetheless, the behaviour pattern exhibited by the tissue would be interesting to investigate further, for example knowing whether oral challenge with PrP^{Sc} causes or not endoplasmic reticulum, namely in PP. In the case of BSE, the experiments were mainly made in bovine cattle and were also scarcer. Also, it is interesting to point out that while CWD and scrapie seem to have the same pathogenesis, in terms of structures infected and age susceptibility(169), BSE-associated transmissible spongiform encephalopathy seems to behave differently upon infection of a new host (198). The differences observed are mainly in sites of accumulation, while BSE-associated PrP^{Sc} tends to accumulate directly in the CNS, scrapie-associated PrP^{Sc} exhibits high accumulation in lymphoid tissue prior to neuroinvasion (156,170,198). The differences observed between different disease-causing PrP^{Sc} render comparison between them

difficult, however some similarities are observed such as the importance of PP in PrP^{Sc} uptake and accumulation, in particular ileal Peyer's Patches (161,170,199).

Still on prion uptake from the gut, the closest animal to humans studied were primates and only one reference has surfaced (181). Furthermore, observations made in the study provide essential clues on prion behaviour in primates, for example the study revealed that, in primates lymphoid tissue invasion in tonsils and spleen by PrP^{Sc} happened only in late-stage incubation or at early clinical signs of BSE. If the same type of pathogenesis is confirmed in humans affected with vCJD then studies carried out in tonsillectomies for purposes of assessing the prevalence of population carrying the vCJD-associated PrP^{Sc} is largely underestimated (200).

Given this, it is important to further study prion behaviour in human and non-human primates to fully understand how these pathogens function in human population. However, it is important to emphasize that given the low prevalence of prion disease in humans, its rapid progression to death after diagnosis, and the high level of biosafety required to study this type of disease it is complicated to this day to study human prion disease on a large scale (112,201).

Within the tag prion/misfolded protein uptake from the gut, references were also extracted mentioning Parkinson's Disease and Alzheimer Disease. These studies, however few in number, reveal that prion diseases and the neurodegenerative diseases herein mentioned have a commonality, they can originate in the gut. Furthermore, they all seem to use the enteric nervous system as a pathway to the CNS (183–187,190).

This study comprises a set of limitations which include the wide variety of labels covered, making the presentation of results lengthier and more difficult to explain, even more so given the large quantity of selected articles for this review (102). In addition to this not all articles were discussed or presented in this work, leaving parts of the data still untreated. However, this study also comprises a set of strengths including the fact that reviews comparing prion diseases and neurodegenerative proteinopathies have mostly directed their focus onto protein misfolding (120,130). In this matter however, PD is the exception with an increasing amount of literature pointing to a clear gut-to-brain phenotype of the disease (119). The clear impact of the gut, its microbiome, and its associated lymphatic and nervous system on these types of disease is another common point that should be further studied in the future with the aim understanding if these commonalities exert a potential for future treatment and earlier diagnosis (136,137,157,186,190,192,202–205). Furthermore, mucosal vaccination appears as a

promising way to delay symptom onset of prion diseases in studies found in this review(206,207), further pressing the importance of uncovering the mechanisms by which both prion and "prion-like" diseases travel from the lumen of the gastrointestinal tract into the central nervous system.

To conclude, this work emphasizes the increasing amount of evidence linking prion diseases and neurodegenerative proteinopathies, while tackling the importance of the gut-brain axis which has demonstrated in recent years to have a major role in both health and disease (123,208–210).

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