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ABSTRACT

The novel antibiotic-exploiting strategy in the treatment of Alzheimer's (AD) and Parkinson's (PD) disease has emerged as a potential breakthrough in the field. The research in animal AD/PD models provided evidence on the antiamyloidogenic, anti-inflammatory, antioxidant and antiapoptotic activity of tetracyclines, associated with cognitive improvement. The neuroprotective effects of minocycline and doxycycline in animals initiated investigation of their clinical efficacy in AD and PD patients which led to inconclusive results and additionally to insufficient safety data on a long-standing doxycycline and minocycline therapy in these patient populations. The safety issues should be considered in two levels; in AD/PD patients (particularly antibiotic-induced alteration of gut microbiota and its consequences), and as a world-wide threat of development of bacterial resistance to these antibiotics posed by a fact that AD and PD are widespread incurable diseases which require daily administered long-lasting antibiotic therapy. Recently proposed subantimicrobial doxycycline doses should be thoroughly explored for their effectiveness and long-term safety especially in AD/PD populations. Keeping in mind the antibacterial activity-related far-reaching undesirable effects both for the patients and globally, further work on repurposing these drugs for a long-standing therapy of AD/PD should consider the chemically modified tetracycline compounds tailored to lack antimicrobial but retain (or introduce) other activities effective against the AD/PD pathology. This strategy might reduce the risk of long-term therapy-related adverse effects (particularly gut-related ones) and development of bacterial resistance toward the tetracycline antibiotic agents but the therapeutic potential and desirable safety profile of such compounds in AD/PD patients need to be confirmed.

Key words:
minocycline, doxycycline, Alzheimer's disease, Parkinson's disease, bacterial resistance, gut microbiota

Statements and Declarations

- The submitted manuscript has not been published before and it is not under consideration for publication anywhere else.

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Author contributions

Iva Markulin and Marija Matasin performed the literature search and participated in writing; Victoria Erdeljic Turk participated in writing and critical reading; Melita Salkovic-Petrisic had the idea for the article, drafted and critically revised the manuscript. All authors read and approved the final manuscript.

1. Introduction

Neurodegenerative diseases (NDs) refer to a broad spectrum of chronic disorders, characterized by a progressive loss of anatomical integrity, structure and function of neurons in the central nervous system (CNS), which leads to inability to function or perform daily tasks, associated with a mental impairment and/or motor dysfunction (Chen et al. 2016; Dugger et al. 2017). NDs demonstrate a specific vulnerability of the selective neurons in the particular brain regions, allowing their classification according to the anatomical localization of the pathological process in CNS which is consequently reflected to the clinical manifestation of the disease (Kovacs 2019). Alzheimer's disease (AD) and Parkinson's disease (PD) are most common NDs. According to 2019 Alzheimer's disease facts and figures, around 5.8 millions of people in United States are living with AD. Only 200 000 patients are younger than 65 years and 81% of patients are 75 years and older (Hebert et al. 2013). The Framingham Heart Study performed a lifetime risk assessment (LTR) for the development of dementia / AD, depending on gender. The LTR at age 45 was 1 in 5 for women and 1 in 10 for men, respectively. Furthermore, although cumulative incidences were similar, LTR was significantly higher in women 85 years of age and older (Chêne et al. 2015). According to Niu and coworkers, the prevalence of AD in Europe is 5.05% and is higher in women than in men (3.31% in men, and 7.13% in women) and increases with age. The incidence of AD in Europe according to the same study is 11.08 per 1000 person-years (Niu et al. 2017). For comparison, according to the World Alzheimer Report from 2015 more than 46 million people all around the world suffer from dementia and more than half of this number refers to AD. It has been estimated that this number will double every 20 years, meaning that in 2050 there will be more than 130 million people with dementia in the world. According to US data related to AD in 2019 approximately 5.8 million Americans are living with it, out of which about 200 000 are younger than 65 years and have early onset form of AD (Prince 2015). According to Matthews and coworkers, in 2014, approximately 5 million people over the age of 65 in the United States lived with Alzheimer's disease or related dementias (ADRD), accounting for 1.6% of the total population. By 2060, that number is expected to double, when the percentage of the population with ADRD will be 3.3%, or 13.9 million people over the age of 65 (Matthews et al. 2019).

Dementia is known as a disease of the elderly population, with a frequency increasing after 65 years of age. As the entire population ages due to an increasing life quality and medical care, an increasing proportion of people with dementia are to be expected in clinical practice. It is also important to emphasize the impact of dementia not only on life of the patients and their caregivers but as well on the society, health system and economy in general (Prince 2015). Parkinson's disease is the second most common cause of dementia worldwide, after AD (Elbaz et al. 2015; Jagmag et al. 2016). According to the European Parkinson's Disease Association (EPDA) and our literature search, there are no papers that would accurately and precisely determine the prevalence of Parkinson's disease globally. It is only possible to make prevalence estimates based on individual reports, papers, and statistics. Global Burden of Disease Study (2016) published that around 6.1 million people worldwide suffer from Parkinson's Disease. In 1990 there was 2.5 million people globally with PD, from which the trend of increasing prevalence can be seen. The increase in frequency is primarily associated with population aging, but environmental factors also play a major role in the disease pathogenesis (Dorsey et al. 2019). PD is more common

in men than in women, with a small-to-frame ratio of about 1.5 (Dorsey et al. 2019; Elbaz et al. 2015; Taylor et al. 2007). Pringsheim and coworkers conducted a systematic review and meta-analysis to detect the prevalence of Parkinson's disease. The results of the meta-analysis confirmed an increase in the prevalence of PD with age - for example, at the age of 40 to 49, the prevalence was 41 per 100,000, and 1,087 per 100,000 at the age of 70 to 79 years. Likewise, a geographical comparison of prevalence was made showing a lower prevalence of PD aged 70 to 79 years in Asia compared to Europe, North America, and Australia. Furthermore, a higher prevalence of PD was shown in men than in women, in all age groups (Pringsheim et al. 2014).

The main problem with AD as well as with other NDs is the fact that they are incurable diseases and drugs available on the market do not act causally nor as disease-modifying therapy but can only relieve the symptoms to a certain extent. The lack of drugs for the symptomatic therapy is particularly problematic in the treatment of AD for which only four drugs are currently available on the market: donepezil, rivastigmine, galantamine and memantine (Van Marum 2009). Despite of enormous efforts and resources invested, ever since then, pharmaceutical industry was unsuccessful in developing new drugs indicated for the AD treatment until aducanumab, a drug recently conditionally approved by the Food and Drug Administration under the accelerated approval pathway based on the drug's effect on a surrogate endpoint (reduction of amyloid ß plaques) that requires to verify the drug's clinical benefit in a post-approval trial (https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug). Due to all mentioned above, a new trend of repurposing the drugs already approved in other indications for targeting the common pathophysiological mechanisms underlying AD and PD, has recently emerged in NDs research, putting into focus also the efficacy of several antibiotics as possible anti-AD/PD agents (Yiannopoulou et al. 2013; Huang et al. 2020). The aim of this review article is to resume the cost-benefit analysis of such a strategy and draw attention to the risks of widespread and long-standing antibiotic administration, especially emphasizing the threat of developing resistant bacterial strains in the context of prevalence and need for a long-term daily treatment of AD and PD as the most common NDs.

2. Common pathophysiological mechanisms in neurodegenerative disorders

AD presents clinically with memory impairment, behavior problems, disorientation, self-care limitations and inability to function independently on a daily basis (Huang et al. 2016; Teo et al. 2019). As the second most common ND, idiopathic PD clinically presents primarily by motor dysfunction (tremor, rigidity, impaired balance, and loss of spontaneous movement) but also by a wide range of non-motor symptoms like dementia and changes in personality, sleep and mood (Pfeiffer 2016). Specific AD hallmarks are pathological accumulation of amyloid-β (Aβ) and tau protein in formation of extracellular plaques and intracellular neurofibrillary tangles in hippocampus and neocortex, respectively, while those of PD are loss of dopaminergic neurons in the substantia nigra pars compacta and their terminals in the striatum, often accompanied by intracellular accumulation of the misfolded α-synuclein protein known as Lewy bodies (Kovacs 2019). In addition to these major pathohistological characteristics, there are some other common mechanisms underlying AD and PD pathophysiology at the cellular level among which most attention has been payed to oxidative stress, mitochondrial dysfunction, apoptosis and neuroinflammation (Huang et al. 2016; Teo et al. 2019) found associated also with other NDs (Gandhi et al. 2012).

Recent literature points also to the insulin resistance in the brain as a common pathological feature shared to a different extent by AD and PD (Barilar et al. 2020).

Pathological aggregation of misfolded proteins. One of the major pathological hallmarks of NDs is aberrant folding of a certain protein which enables exposure of its hydrophobic amino acids possessing the affinity to self-assemble into oligomers, larger aggregates and fibrils, and to interact with other cellular proteins and cellular membranes finally resulting in cell death (Holmes et al. 2014). Diseases like NDs in which misfolding results in the conversion through aggregation of normally soluble proteins into intractable, highly stable beta sheet amyloid structure are of a particular interest due to currently non-existing causal or disease-modifying therapy (Dobson et al. 2020).

Amyloids are a special type of protein aggregates. Approximately 60 heterogeneous proteins have been identified as capable of forming amyloids, and about 30 of them are associated to human diseases including NDs, like Aβ and tau protein in AD, α-synuclein in PD, prion protein (PrP) in Creutzfeldt–Jakob disease (CJD), huntingtin in Huntignton disease (HD), superoxide dismutase-1 (SOD1) and TDP-43 in Amyotrophic lateral sclerosis (ALS) (Avila et al. 2017). Literature data suggests that the toxic species in amyloid pathology are more likely to be prefibrillar aggregates than mature amyloid fibrils which is why cell protection from soluble oligomeric amyloid species can be achieved by the passage of these toxic forms to harmless insoluble fibrils (Avila et al. 2017). Pathological deposition of misfolded proteins with β-sheet amyloid conformations has been found as a possible trigger of deleterious effects such as neuroinflammation, mitochondrial dysfunction, oxidative damage and altered membrane permeabilization.

Oxidative stress and mitochondrial dysfunction. A growing body of evidence suggests a major role of oxidative stress in the development of AD (Cheignon et al. 2018; Kamat et al. 2016; Tönnies et al. 2017) and PD (Picca et al. 2020). Definition of oxidative stress is imbalance between production and utilization of reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide (H2O2), hydroxyl radicals and hydroxyl ions. Electron transport chain (ETC), which happens on the neuronal inner mitochondrial membrane, is the place of ROD origin, as well as in cells like astrocytes and microglia in CNS. As mitochondria are the main source of ROS, mitochondrial dysfunction has a major contribution to oxidative stress development. The role of ROS in the onset of NDs is the risk of damaging cell structures, specially macromolecules like nucleic acids, lipids, proteins, as well as cell membranes (Persson et al. 2014).

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) enzymes are involved in transfer of electrons across the plasma membrane to molecular oxygen, and generation of the superoxide anion and subsequently ROS, H2O2 and hydroxyl radicals (Tarafdar et al. 2018). Additionally, generation of ROS by the activation of NOX2 is commonly combined with large nitrogen oxide (NO) production and the accumulation of peroxynitrate causing reactive nitrosative species (RNS) (Brown 2007). Brain itself, specially neurons and astrocytes, consumes high values of oxygen, and consists of high percentage of lipids (Gandhi et al. 2012). Due to that it is susceptible to damage caused by ROS and RNS particularly in ageing and age-related diseases like AD and PD (Luo et al. 2020; Trist et al. 2019).

Numerous studies have pointed to the increased markers of oxidation in brain of AD patients (Ahmad et al. 2017; Galasko and Montine 2010; Mantzavinos and Alexiou 2017; Skoumalová and Hort 2012; Wojsiat et al. 2017). Many brain cell membranes have phospholipids composed of polyunsaturated fatty acids and due to are more sensitive to oxidative stress caused by free radicals. Double bonding allows the removal of hydrogen ions and enhanced lipid peroxidation, the prominent features of degenerative alterations occurring in brain (Tsaluchidu et al. 2008). Aβ seems to play a dual role in regard the oxidative stress. It acts like an antioxidant in physiological conditions by blocking the formation of hydroxyl radicals and thus preventing protein and lipid oxidation in mitochondria (Sinha et al. 2013). However, pathological Aβ1-40 and Aβ1-42 fragments which dominate in AD condition demonstrate pro-oxidative activity confirmed by detection of increased markers of protein oxidation and lipid peroxidation in primary neuronal culture, cortical synaptosomes and astrocytes (Butterfield et al. 2018). Deposition of Aβ plaques causes damage also to mitochondria with ROS production disrupting the electron transport chain (Swerdlow et al. 2010). Accumulated Aβ facilitates the expression of the receptor for advanced glycation end products (RAGE) in microvascular endothelial cells neurons and microglia, which induces ROS mainly through the activity of NADPH oxidases (Piras et al. 2016). Aβ accumulation activates NADPH oxidase in astrocyte cells, but with different mechanism than in microglia, referring to induction of calcium entry into them (Abramov et al. 2004).

Oxidative stress and generation of ROS play a significant role also in PD pathophysiology; elevated levels of oxidized lipids, proteins, and DNA, along with reduced levels of glutathione are found in the substantia nigra of PD patients (Gandhi et al. 2012). Activated microglia release free radicals which cause uncontrolled neuro-inflammatory responses, and damaged dopaminergic neurons release oxidized molecules such as neuromelanin and α-synuclein which further activate microglial cells (Tarafdar et al. 2018). Additionally, RNS also plays important role in nitrosative stress in PD; nitric oxide (NO) produced by neuronal or inducible isoform of nitric oxide synthase (nNOS or iNOS) is found in large quantities intracellularly as well as extracellularly around dopaminergic neurons (Tieu et al. 2003). NO mediates lipid peroxidation and negatively affects complex I and IV of the mitochondrial electron transport chain in PD brain (Singh et al. 2019). Additionally, mitochondrial dysfunction in PD is closely related to the increased ROS formation and Complex I deficiencies of the respiratory chain account for the majority of unfavorable neural apoptosis generation in this disease (Schapira et al. 1990).

Apoptosis. Apoptosis normally occurs as a homeostatic mechanism to maintain cell populations in tissues or as a defense mechanism when cells are damaged by disease or noxious agents. However, NDs are associated with inappropriate apoptosis. There are two main apoptotic pathways (the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway) which converge on the execution pathway initiated by the cleavage of caspase-3 and subsequently resulting in the irreversible processes leading towards cell death (Elmore 2007). The extrinsic signaling pathways are initiated by death signal proteins tumor necrosis factor alpha (TNFα) and Fas ligand and the intrinsic ones by a release of mitochondrial cytochrome c and pro-apoptotic Bcl-2 family members of Bax, Bad and Bid. In AD condition, Aβ may induce apoptosis by inducing oxidative stress as previously mentioned, and by triggering increased Fas ligand expressions in neurons and glia; activation of microglia results in TNFα secretion and triggering of a downstream signaling cascade leading to apoptosis (Ethell and Buhler 2003).

Apoptosis seems to be one of the main mechanisms of neuronal loss also in PD but the predominant mechanism is thought to be the intrinsic apoptotic pathway; mitochondrial dysfunction and a defect in the activity of mitochondrial complex I were found as an early occurrence in PD patients and animal PD models (Erekat 2018). Nigral dopaminergic neurons are particularly susceptible to dysfunction of mitochondrial complex I and dopamine metabolism leads to ROS generation, which all together may lower the threshold for apoptotic cell death contributing to dopaminergic neuronal death through apoptosis (Erekat 2018).

Neuroinflammation. Neuroinflammation is defined by elevated levels of pro-inflammatory cytokines in nerve tissue which can be induced by different factors and pathological processes, like ageing, trauma, infections, drugs as well as diseases like NDs (Barrientos et al. 2015). Pro-inflammatory cytokines associated with promotion and deterioration of NDs are interleukin (IL)1β, IL-6, interferon-gamma (IF-γ) and macrophage migration inhibitory factor (Singhal et al. 2014). An acute neuro-inflammatory response aims to minimize the brain injury regardless its cause and is beneficial to the CNS, while the chronic inflammation is associated with the long-standing activation of microglia and sustained release of inflammatory mediators, which eventually lead to an increase of oxidative and nitrosative stress and endless stimulation of the inflammatory cycle with detrimental consequences (Chen et al. 2016). Neuroinflammation in NDs encompasses the action of different types of cells, from nerve, immune and inflammatory through different signaling mechanisms and inflammatory mediators (Kempuraj et al. 2016) and leads to tissue damage, altered neurotransmitter function and neuronal dysfunction (Giovannini et al. 2002). Microglial cells are the major source of pro-inflammatory cytokines. They act as a link or mediator between the nervous and immune systems. Due to ageing, its function shows signs of impairment and chronic inflammation, leading to induction of neurodegeneration (Zilka et al. 2012). Practically all the cytokines and chemokines that have been studied in AD, including IL-1β, IL-6, TNF-α, IL-8, transforming growth factor-β (TGF-β), and macrophage inflammatory protein-1α (MIP-1α), seem to be upregulated in AD human brain (Holmes 2017). IL-1-positive activated microglia is co-localized with Aβ plaques and neurofibrillary tangles in AD while astrocytes express the receptors for binding inflammatory cytokines and chemokines, and additionally iNOS which contributes to NO-mediated toxicity (Calsolaro and Edison 2016). Considering the neuroinflammation in PD, activated microglial cells and infiltrating T lymphocytes have been fund in the human substantia nigra (Brochard et al. 2009; McGeer et al. 1988). Debris of degenerating neurons and neuromelanin might represent activating agents for microglial cells in PD as well as α-synuclein whose misfolded form transiting from one neuron to another in a prion-like mechanism might be at the origin of the self-perpetuation of neuroinflammation (Hirsch et al. 2021). Increased levels of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6, and decreased levels of neurotrophins such as brain-derived neurotrophic factor (BDNF), have been found in the nigrostriatal region of postmortem PD patients (Nagatsu et al. 2005).

However, despite of the above mentioned possible triggers, it is still not fully understood whether neuroinflammation is a driven force of neurodegenerative disorders or is it a consequence of the metabolic dysfunction occurring earlier in the progression of diseases (Yin et al. 2016).

Brain insulin resistance. Central metabolic changes characterized by insulin resistant brain state (IRBS) and glucose hypometabolism have been found associated with both AD (De Felice et al. 2014; De La Monte et al.

2014; Hoyer 2004) and PD (Athauda and Foltynie 2016; Yang et al. 2018), possibly as a common shared pathophysiology (Barilar et al. 2020). Glucose metabolism, insulin, insulin receptors (IR) and insulin-like growth factors in the brain significantly contribute to the neuronal development and brain functioning. Among other functions, brain insulin provides neuronal survival, participates in synaptic plasticity, has an important role in regulation of memory, learning and cognition, and also participates in neuronal glucose metabolism (Banks et al. 2012). Olfactory bulb, hypothalamus, cerebral cortex and hippocampus are brain regions with a highest IR density. Binding of insulin to IR triggers its tyrosine kinase activity and the downstream signaling cascade which involves two main signaling pathways; phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) pathway, respectively (Hoyer 2002). IR-PI3K/Akt pathway further targets multiple downstream pathways including the glycogen synthase kinase-3 (GSK3), which beside other roles, regulates the intraneuronal glucose uptake, homeostasis od tau protein phosphorylation and processing of amyloid beta precursor protein (Ma 2014), and mammalian target of rapamycin complex 1 (mTORC1), a major regulator of cellular proliferation and survival (Stretton et al. 2015). IR-MAPK pathway plays a direct role in cell proliferation, differentiation and gene expression and contributes to the normal function and survival of neuronal cells (Banks et al. 2012). Because of the complexity of these insulin-regulated processes involved in important brain functions, it is to be expected that impaired insulin function and IRBS development is strongly associated with neurodegenerative diseases (Cai et al. 2012; Craft and Watson 2004; De La Monte 2017). Decreased mRNA/protein expression of IR, decreased IR-tyrosine kinase activity accompanied by decreased insulin receptor substrate-1 (IRS1), and PI3K/Akt levels as well as increased serine (inhibitory) phosphorylation of IRS-1 and increased (harmful) GSK3 activity have been reported in the brain of AD patients post-mortem (Frölich et al. 1998; Steen et al. 2005). Additionally, lower concentrations of insulin in cerebrospinal fluid (CSF) and higher plasma insulin concentrations accompanied by lower CSF/plasma insulin ratio, have been found in demented patients (Craft et al. 1998) while intranasal insulin administration leads to improvement in cognitive functions in certain population of AD patients (Avgerinos et al. 2018). Therefore, a lot of pathological abnormalities in AD might be a consequence of IRBS, but it has to be mentioned that their interaction seems to be a bidirectional one since Aβ itself is capable of impairing the insulin transmission in AD brain at different levels (de la Monte 2017) as well as that IRBS and impaired glucose utilization might be consequences of chronic inflammatory processes and oxidative stress, not only in brain but in the entire organism (Giovannini et al. 2002; Maciejczyk et al. 2019; Rao et al. 2011). Decreased expression of insulin and IR as well as increased expression of markers of IRBS have been found also in the brain of PD patients (Moroo et al. 1994; Takahashi et al. 1996; Tong et al. 2009), and like for AD, literature data suggests that the interaction is bidirectional, and that also misfolded α-synuclein might drive defective signaling in PD (Athauda and Foltynie 2016).

One should not forget that aging itself is associated with increased tendency to development of chronic diseases and is considered to be a major risk factor for NDs due to the age-related degenerative changes and genomic instability (Chow et al. 2015; Hou et al. 2019) and epigenetic changes like methylation (Bradley-Whitman and Lovell 2013) but also because of the age-related decrease in brain insulin level and IR expression (Akintola and van Heemst 2015) and vulnerability to oxidative stress (Liguori et al. 2018).

3. Tetracyclines as a potential novel strategy in the treatment of neurodegenerative disorders

Repurposing of drugs already approved for their use in humans in other indications like anti-inflammatory drugs or antioxidant agents, has become one of the new strategies in a research of a disease-modifying therapy for AD and PD. In this respect, the multifactorial characteristics underlying the NDs pathophysiology suggest that multitarget drugs would be more appropriate than those possessing, for example only anti-inflammatory, antiapoptitic or antioxidant activity alone. Consistent with the recent findings that some antibiotic drugs demonstrate additionally one or more of these desirable activities (Appleby et al. 2013; Garrido-Mesa et al. 2013; Kaeberlein and Galvan 2019), extensive investigations of tetracyclines as possible new ND-modifying drugs have been initiated (Balducci and Forloni 2019; Molloy et al. 2013; Yimer et al. 2019; Yulug et al. 2018). Tetracyclines have been chosen particularly due to the structural analogies of tetracycline with Congo red (an azo dye used for specific detection of pathological Aβ aggregation seen as apple-green birefringence under polarized light) (Ladewig 1945), and iododoxorubicin (an anthracycline anti-cancer drug which inhibits the formation of amyloid aggregates in vitro and in vivo) (Merlini et al. 1995; Tagliavini et al. 1997). The compounds share the characteristic of containing an extended hydrophobic core formed by aromatic moieties with a large number hydrophylic substituents conferring an amphiphilic character (Tagliavini et al. 2000).

Tetracyclines are broad-spectrum bacteriostatic antibiotics active against a variety of gram-positive, gram-negative and atypical bacteria. Their antibiotic mechanism of action includes inhibition of bacteria protein synthesis by reversible binding to 30S ribosomal unit with a consequent prevention of the aminoacyl tRNA from binding to the ribosome (Ian and Marilyn 2001). Among the existing groups/generations of tetracyclines (the tetracycline natural product, tetracycline semi-synthetic derivatives and chemically modified tetracyclines), the most clinically used as antibiotic drugs are semi-synthetic compounds doxycycline and minocycline (Fuoco 2012). The chemical structure reveals linearly fused tetracyclic nucleus composed of A, B, C and D rings to which different functional groups and chains are attached in the upper and lower peripheral zones (Bortolanza et al. 2018; Stoilova et al. 2013) (Fig. 1). The dimethylamine (DMA) group at C4 carbon in A ring (upper zone) is necessary for the antibacterial activity of tetracyclines while modifications in D ring at the positions of C7 to C9 may influence other biological actions of tetracyclines (Chen et al. 2012). The lower peripheral region contains functional groups that are responsible for the chelation of metal ions (Bortolanza et al. 2018). Modifications of the upper peripheral are involved in alterations of the basic physicochemical characteristics such as polarity, electronic configuration and charge density and distribution, resulting in changes in solubility and lipophilicity (Stoilova et al. 2013). There are minor differences in the chemical structure between the tetracycline agents; comparison of tetracycline, doxycycline and minocycline demonstrates that minocycline differs from the other two in that diethylamino group is substituted at C7 in D ring and in lacking a functional group at C6 (C ring) while doxycycline differs from tetracycline in that OH group is not substituted at C6 position in C ring (Chen et al. 2012).

Absorption after oral administration of tetracyclines occurs mainly in stomach and upper parts of small intestine, and reaches approximately 95 -100% for doxycycline and minocycline. It is impaired by multivalent cations in drugs (e.g. antacids) and dairy products (calcium) which chelate tetracyclines and form non-absorbable compounds in gastrointestinal tract (absorption of doxycycline is less affected by food). Doses of doxycycline and

minocycline usually used to treat infections caused by susceptible microorganisms are in range 100 – 200 mg/day; peak blood levels of approximately 2-4 μg/ml are achieved with a 200 mg dose 2-3 hours after dosing with an elimination half-life of 15-30 hours (Beauduy 2018). They are mostly excreted by the kidneys through the process of glomerular filtration but partly also by bile/feces (Ian and Marilyn 2001).

The penetration capacity of antibiotic drugs into the CNS depends on many factors such as the molecular size, electric charge, lipophilicity, plasma protein binding, affinity to active transport systems, present inflammation and pathology of the subject and also CSF flow (Nau et al. 2010) Most of the previous experience with tetracyclines in the treatment of CNS infection caused by susceptible microorganisms refers to doxycycline which, because of its good lipid solubility and passing of the blood brain barrier (BBB), has been considered as a preferential choice of tetracycline drugs in such indications (Dotevall and Hagberg 1989; Karlsson et al. 1996). Comparison of tetracycline, doxycycline and minocycline reveals that minocycline has the highest lipophilicity among these three tetracycline drugs (0.025, 0.600 and 1.100, respectively) which contributes to the fact that minocycline crosses the BBB much more readily than doxycycline (or tetracycline) (Colaizzi and Klink 1969). However, due to more favorable safety profile in CNS side effects, doxycycline seems to remain the preferred oral antibiotic for treatment of the indicated CNS infections, for instance Lyme neuroborreliosis (Dotevall and Hagberg 1999; Macdonald et al. 1973).

There are only few reports on pharmacokinetics of tetracyclines in rodents, the species most frequently used in preclinical research of therapeutic potential of tetracyclines in models of NDs. The elimination half-life of doxycycline was found to be significantly faster in mice and rats than in humans (3-6 vs 15-30 hours) (Lucchetti et al. 2019; Wittenauet al. 1971). This data indicates that a single daily dose treatment regimen may be justified in humans but not in rodents, which should also be taken in account in animal-to-human data translation. Considering the differences in pharmacokinetics of rodents and human, more accurate comparative data can be provided based on the drug plasma concentration than the dose itself, and available pharmacokinetic rodent studies provide only doxycycline pharmacokinetic data. In human, oral doxycycline doses of 100 – 200 mg/day lead to drug plasma concentrations of approximately 1.7 – 5.9 μg/ml (Agwuh and MacGowan 2006) and dose of 40 mg/day produces a plasma concentration around 0.2 – 0.5 μg/ml (Mckeage and Deeks 2010) while in rats treated chronically with oral doses of 75 and 200 mg/kg, detected drug plasma concentration was on average 0.14 and 0.32 μg/ml, respectively (Chtarto et al. 2016). On the other hand, intraperitoneal injections of 10 and 100 mg/kg in young healthy mice produced plasma concentrations ranging from 2 -10 μg/ml that were superimposable with human oral doxycycline doses of 100 – 200 mg (Lucchetti et al. 2019). Comparative pharmacokinetic study of doxycycline was performed in wild-type and transgenic APP23 mice AD model after intraperitoneal administration of single and repeated injections of 10 and 100 mg/kg (Lucchetti et al. 2019). Briefly, there were no differences in doxycycline concentrations in the brain between these two group of mice, and there were no differences between mice and human pharmacokinetic parameters in plasma concentrations as well as in brain-to-plasma ratio (mice) vs cerebrospinal fluid/serum ratio (humans) (Lucchetti et al. 2019). Comparing the mice and human data based on the similar plasma values obtained following 10 and 100 mg/kg/day dose in healthy mice and 100 mg/day in patients with CJD, mean maximum brain concentrations of 0.24 and 1.45 μg/g of brain were found in mice and 0.6 – 3.0 μg/g of brain postmortem in patients with CJD (Lucchetti et al. 2019). Expressed

as a brain-to-plasma ratio of doxycycline concentration approximate value of 0.2 was found in mice (both wild and transgenic mice) while 26% drug penetration from blood to cerebrospinal fluid was reported in humans with latent neurosyphilis (Yim et al. 1985; Lucchetti et al. 2019).

3.1. Minocycline and doxycycline non-antibiotic actions in general

Independent of their antibiotic activity, minocycline and doxycycline have been found to possess several non-antibiotic effects which have drawn attention as possible mechanisms that might provide a neuroprotection needed to cope with the commonly shared pathophysiology of NDs. Considering the pleiotropic biological effects elaborated below, it is not clear yet what makes the tetracyclines efficient against so different targets but it has been proposed that increased variability in conformational changes related to the surrounding conditions and consequently structural flexibility (extended and folded conformation of the molecules) might be at least partly responsible for that (Stoilova et al. 2013).

Antiamyloidogenic activity. Large body of evidence suggests that tetracyclines have antiamyloidogenic activity by interfering with the aggregation process of amyloidogenic proteins (Stoilova et al. 2013). The facts that tetracycline resembles to iododoxorubicin and the ability of the latter drug to produce clinical benefits in patients with immunoglobulin light-chain amyloidosis initiated the research on the efficacy of tetracycline in preventing the pathological aggregation and propagation of PrP among the first studies showing the tetracycline antiamyloidogenic property. Pathological PrP^{Sc} isoform is capable of converting the normal host-encoded protein into altered form - amyloid cross-β conformation (Prusiner et al. 1998). In vitro study confirmed binding of tetracycline to synthetic peptides homologues to residues 82-146 and 106-126 of human PrP protein, and demonstrated that tetracycline markedly prevented the formation of PrP peptide macroaggregates (Tagliavini et al. 2000). Furthermore, the same study showed that tetracycline reverted the protease-resistance of PrP^{Sc} isolated from brain of patients with CJD, and additionally, that tetracycline was capable of preventing the PrP-induced neuronal death and astrocyte proliferation in vitro (Tagliavini et al. 2000). Tetracycline structure, in particular the nature and position of polar substituents, seems to play an important role in the interaction with hydrophobic domains of PrP peptides interfering with the conversion process and/or PrP^{Sc} assembly (Tagliavini et al. 2000). Forloni and coworkers performed in vitro experiments in cell-free medium to identify the PrP regions involved in conformational conversion of PrP into PrP^{sc} by using synthetic peptides homologous to residues 106-126 and 82-146 (Forloni et al. 2012). The tetracycline derivatives were shown to destabilize the structure of preformed PrP 106-126 fibrils, also reducing their resistance to proteinase K digestion. In vitro incubation of PrP^{Sc} from patients with sporadic CJD with tetracycline and doxycycline (10 μM – 1 mM) for 48h resulted in a dose-dependent decrease in proteinase K resistance of PrP^{Sc}, which reached almost 80% at the highest drug concentration (Forloni et al. 2009). The activity of minocycline to reduce the proteinase K resistance was lower against PrP 106-126 peptide than against the purified human PrP^{Sc} possibly due to a lack of C terminal domain in the former, previously shown to be also involved in the drug-PrP interaction (Forloni et al. 2009). Furthermore, it has been shown that although the C-terminal of PrP is important for the peptides' fibrillogenic activity, it has no major role in the PrP's neurotoxicity, due to the authors hypothesized that the neurotoxic activity of PrP peptides is linked to the oligomeric species rather than amyloid fibrils, and thus antitoxic properties of tetracyclines on PrP peptides can

be attributed their ability to bind and neutralize small peptide aggregates (Forloni et al. 2009). The same group reported that in human neuroblastoma cells expressing PrP H187R which, in contrast to the PrP wild type, is not transported to the cell membrane but accumulates intracellularly, doxycycline dose-dependently facilitated PrPH187R transport to the cell surface and thus the correct folding of the mutated protein (Forloni et al. 2009).

The successfulness of antiprion activity of tetracycline initiated further research on tetracyclines activity against other amyloidogenic proteins like transthyretin, β2-microglubulin amyloid fibrils and immunoglobulin light-chain, and, most importantly, against Aβ and α-synuclein as the AD and PD neuropathological hallmarks, respectively (Cardoso et al. 2006; Giorgetti et al. 2011; Stoilova et al. 2013; Ward et al., 2011). It has been proposed recently that the binding mode of tetracyclines to the misfolded proteins seems to be dependent on the conformation of the aggregation-prone segment rather than on its sequence explaining thus the capability of tetracyclines to inhibit amyloid aggregation of diverse amyloidogenic proteins (González-Lizárraga et al. 2020).

MINOCYCLINE. Minocycline efficacy was explored in oculoleptomeningeal amyloidosis (OA) (considered as a form of cerebral amyloid angiopathy) that belongs to transthyretin (TTR)-related amyloidosis associated with slow progressive dementia, ataxia, seizures and recurrent subarachnoidal hemorrhaging. Three-day pretreatment with minocycyline was capable of preventing memory deficits and reversing neuroinflammation in mice model of OA induced by intracerebroventricular injection of amyloid fibrils composed of A25T TTR but no direct effect on TTR was detected Azevedo et al. 2013). Considering the drug anti- Aβ activity, in vitro study of Familian and coworkers demonstrated that minocycline dose-dependently inhibit Aβ1-42 fibril formation in cell culture of adult microglia cells isolated from brains of patients with AD post-mortem (the effect was associated with inhibition of human microglial activation) (Familian et al. 2006). Oral minocycyline treatment (50 mg/kg/day) in diet-induced hyperinsulinemic and streptozotocin-induced diabetic rats initiated 2 days after diabetes induction and continued for 4, 6 and 8 weeks, resulted in decreased (50%) expression of Aβ1-40/1-42 proteins but not that of amyloid precursor protein in the brain (Cai et al. 2013). In primary cortical neurons treated with Aβ1-42, minocycline treatment prevented Aβ-induced neuronal death, and additionally lowered generation of caspase-3-cleaved tau fragments (Noble et al. 2009).

DOXYCYCLINE. Systemic amyloidoses are progressive protein misfolding diseases and their most common form is monoclonal immunoglobulin light chain amyloidosis (AL amyloidosis), caused by the conversion of immunoglobulin light chains from their soluble functional states into amyloid fibrillary aggregates (Merlini et al. 2018). Doxycycline has been proven successful in AL amyloidosis both in inhibiting light chain toxicity in vitro and in animal model of the disease as well as in a retrospective case-matched study where the addition of doxycycline to standard chemotherapy reduced early mortality in cardiac AL amyloidosis (Merlini et al. 2018).

Considering the anti-Aβ activity of tetracycline drugs, Forloni and coworkers were among the first who reported that 5-day co-incubation of synthetic Aβ1-42 with tetracycline or doxycycline (10 μM-1mM) significantly decreased both the formation of amyloid fibrils and the resistance of Aβ1-42 fibrils to trypsin digestion, demonstrating also a de-fibrillogenic effect against pre-formed Aβ1-42 fibrils (Forloni et al. 2001). Doxycycline-induced prevention of Aβ fibrillization and favoring of generation of non-toxic and non-amyloid structures was

also confirmed in a neuroblastoma cell line studies (Costa et al. 2011). This study additionally demonstrated that doxycycline had no effect on the neurotoxicity of pre-aggregated Aβ oligomers, indicating that doxycycline might be incapable of counteracting deleterious effects of already assembled toxic oligomers (Costa et al. 2011). Interaction of doxycycline with early Aβ 1-42 oligomers leading to generation of large non-fibrillary and non-toxic aggregates was reported also by other groups (Airoldi et al. 2011). Recent molecular dynamics study on destabilization of Aβ42 fibrils by tetracycline compounds demonstrated that doxycycline tightly binds the exposed hydrophobic amino acids of the Aβ42 amyloid fibrils, partly leading to destabilization of the fibrillar structure (Gautieri et al. 2019). An extensive in vitro study of Medina and coworkers on the interaction of doxycycline and tau protein was performed by using heparin-induced 2N4R tau fibrillization as well as 4R truncated species that undergo self-aggregation (Medina et al. 2021). They demonstrated that doxycycline inhibited tau aggregation in a dose-dependent manner, showing an IC50 of 29 μM and the optimal inhibition of tau amyloid aggregation exerted at 100 μM, also remodeled tau aggregates affecting the formation of hydrophobic patches and inhibiting tau seeding ability and toxicity for cultured human neuroblastoma cells (Medina et al. 2021). Furthermore, doxycycline treatment diminished ß-structure formation of tau aggregates, and interfered with heparin-induced tau fibril formation by interacting with the microtubule-binding region, inducing novel conformational changes and exposing previously-inaccessible sites to protease cleavage (Medina et al. 2021).

Doxycycline was also found to successfully reshape α-synuclein oligomers into non-toxic high-molecular-weight species with decreased ability to destabilize biological membranes (Avila et al. 2017). Similar finding was confirmed also by a recent study which demonstrated that doxycycline (1 and 10 μM) reduced α-synuclein aggregation in a cell free system as well as in H4, SH-SY5Y and HEK293 cells, where such a treatment also decreased the number and size of α-synuclein aggregates and reduced the level of reactive oxygen species generated by mitochondria (Dominguez-Meijide et al. 2021). Another in vitro research also revealed doxycycline-induced beneficial remodeling of α-synuclein oligomers (González-Lizárraga et al. 2017). Doxycycline (100 μM) was unable to interact with monomeric α-synuclein (preserving thus the monomeric form for its physiological function) but inhibited the assembly of α-synuclein oligomers into larger toxic aggregates. The oligomers formed in the presence of doxycycline did not function as template for the conversion into amyloid fibrils (called "off-pathway" oligomers by the authors). In contrast to "on-pathway" oligomers which have predominantly antiparallel ß-sheet structure, the "off-pathway" α-synuclein oligomers were rich in parallel ß-sheet structure and exposed hydrophobic patches to a lesser extent (González-Lizárraga et al. 2017).

MINOCYCLINE VERSUS DOXYCYCLINE COMPARATIVE STUDIES. Different treatment protocols used to compare the efficacy of tetracycline, doxycycline and minocycline in hamsters with transmissible spongiform encephalopathies demonstrated that tetracycline and doxycycline significantly prolonged the median survival of hamsters, by 32% and 25%, respectively, while minocycline increased median survival by 81% (Forloni et al. 2012). Recent research of Xu and coworkers compared the inhibitory effect of minocycline, methacycline and doxycycline on aggregation of human islet amyloid polypeptide (hIAPP) and Aß whose aggregation represents the pathological hallmarks of type 2 diabetes mellitus and AD, respectively (Xu et al. 2020). The inhibitory ability of the three tetracyclines was stronger against hIAPP than against Aß. However, while the three drugs had similar inhibitory activity against hIAPP, their effects differed against Aß with much stronger inhibitory activity of

minocycline and methacycline compared to doxycycline as consistently seen in different assays used. All three tetracyclines demonstrated concentration-dependent inhibitory effects on the fibril formation, scattered fibrils into monomers and impaired the two amyloid peptide aggregation (Xu et al. 2020).

The group of Smith and coworkers performed a set of experiments to test the ability of tetracycline, doxycycline, and minocycline to reduce aggregation of huntingtin in vitro as well as to explore the therapeutic potential of these tetracyclines in modifying the disease in its animal model in vivo (Smith et al. 2003). While in hippocampal slice culture all three drugs reduced aggregation of the huntingtin up to 10-fold at concentration of 30 uM, in animal HD model in vivo none of the drugs influenced grip strength nor were they capable of reducing aggregate load in the brain post mortem in doses which achieved brain drug concentration in the range of 15 to 20 uM (Smith et al., 2003). The reason for this ineffectiveness of the three tested tetraycyclines against huntingtin-induced pathology in this experiment has not been resolved yet but it stands in contrast to previously reported minocycline-induced delay in disease progression associated with inhibition of caspase-1/-3 expression in R6/2 transgenic HD mouse model (Chen et al. 2000).

Recent in vitro study of Gonzalez-Lizarraga and coworkers has brought very important novel findings on the comparison of minocycline and doxycycline in their interaction with α-synuclein (González-Lizárraga et al. 2020). While doxycycline inhibited α-synuclein aggregation with IC50 of 14.49 μM and halted fibril grow, minocycline failed to inhibit both of these processes (González-Lizárraga et al. 2020). It has been proposed that this difference in interaction could be related to the presence of DMA group in the structure of minocycline (on C-7) and its absence in doxycycline molecule as DMA seemed to obstruct the ability of the tetracycline drug to inhibit α-synuclein amyloid aggregation (González-Lizárraga et al. 2020).

Thus, numerous literature data provides evidence on antiamiloidogenic activities of tetracyclines (and differences between minocycline and doxycycline in this regard) based on their binding to ß-sheet forming domain of different amyloidogenic proteins, affecting the formation of toxic oligomers, inhibiting fibrillogenesis and preventing further fibril growth, disaggregating the mature fibrils and favoring the formation of stable, non-toxic amyloid protein structures (Stoilova et al. 2013). Although a huge progress has been made in understanding the molecular processes in this complex interaction, the exact relationship between the tetracycline drug structure and respective antiamyloidogenic properties still needs some clarification. Additionally, when interpreting the in vitro results it is important to keep in mind the ratio between the particular tetracycline drug and the aggregating protein concentration as most of the studies were performed with this molar ratio being 1:1 while doxycycline achieves brain concentration in the order of μg/mL and amyloidegenic protein concentration is in the order of ng/ml (González-Lizárraga et al. 2017; Socias et al. 2017; Yim et al. 1985).

Antioxidant activity - scavenging of reactive oxygen species. Tetracycline, doxycycline and minocycline contain a multiple-substituted phenol ring which is crucial for their ROS-scavenging abilities since the reaction of the phenol ring with a free radical generates a phenolic radical that is relatively stable and unreactive while the scavenging potency strongly depends on the number and size of phenol ring substituents (Griffin et al. 2010; Stoilova et al. 2013). Due to the presence of the diethylamino group on the phenolic carbon in D ring (Fig. 1), minocycline is 9-250 times more potent than doxycycline and 200-300 times more potent than tetracycline, as a

ROS scavenger (Griffin et al. 2010). Direct scavenging of ROS, superoxide and peroxynitrite as well as H2O2 quenching by minocycline has been shown in several cell-free mixed-radical assays (Kraus et al. 2005). Additionally, minocycline and doxycycline are reported to inhibit oxidative stress also by decreasing protein expression and the enzyme activity of iNOS which results in reduction of NO levels and thus diminishes its reaction with oxygen radicals which otherwise would form cytotoxic species like peroxynitrite (Amin et al. 1997). Some literature data indicates that peroxynitrite might be a direct target of minocycline in vivo (Stoilova et al. 2013).

Antiapoptotic effects and mitochondrial dysfunction. Tetracyclines have been shown to possess also antiapoptotic properties which are believed to involve the reduction of caspase expression, in particular caspase-1 and -3 (Griffin et al. 2010; Stoilova et al. 2013). Minocycline was found to act antiapoptotic at both extrinsic and intrinsic signaling pathways by inhibition of caspase expression and by mitochondrial stabilization. It reduces the expression and activation of the executioner caspase-3 (Chen et al. 2000) but also inhibits pro-apoptotic Ca^{2+}- and Bid-induced mitochondrial cytochrome c release and upregulates antiapoptotic factor Bcl-2 (Wang et al. 2004). At low micromolar concentrations minocycyline impairs several energy-dependent functions of mitochondria in vitro (Kupsch et al. 2009). Its mechanism of mitochondria stabilization is related to its ability to chelate Ca^{2+}, bind to liver mitochondria membranes, partially uncouple mitochondria by formation of ion channels, and to prevent Ca^{2+} accumulation in the mitochondrial matrix (Antonenko et al. 2010; Garcia-Martinez et al. 2010). Antiapoptotic effect of minocycline has been documented in various peripheral tissue (Merati et al. 2018) as well as in the brain (Chen et al. 2012). Recent data indicates that doxycycline exhibits antiapoptotic effects at low concentrations in HeLa cells by inhibition of FasL-induced extrinsic pathway and consequent inhibition of caspase activation which has not been achieved by minocycline and tetracycline at same concentrations (Yoon et al. 2015). Mitochondrial stress induces a cross-talk between the mitochondria and cytosolic proteostasis and doxycycline has been shown to reduce the accumulation of proteins intended for degradation by the proteasome in the mouse brain in the region- and sex-specific manner both in healthy mice subjected to heat shock and SOD1-G93A mice model of ALS (Jenkins et al. 2021).

Anti-inflammatory effects. In general, anti-inflammatory activity of doxycycline and minocycyline is based on various mechanisms which include the above mentioned ROS scavenger effects and antiapoptotic activity but additionally also the suppression of matrix metalloproteinase (MMPs) and inhibition of pro-inflammatory cytokines and pro-inflammatory enzymes like iNOS (Bahrami et al. 2011). MMPs are zinc-dependent proteases involved in proteolysis of the extracellular matrix which contribute to the inflammation process and their upregulation can contribute to demyelinisation, neurotoxicity and neuroinflammation (Yong et al. 2001). Beneficial effects of tetracyclines are demonstrated by a direct inhibition of MMPs activity and the inhibition of their expression (Griffin et al. 2010; Stoilova et al. 2013). Direct inhibition of MPPs is mediated by an interaction between the tetracycline molecule and metal ions within the MMP, and it depends on the tetracycline species, MMP species and the pH (Griffin et al. 2010). Doxycycline has already been marketed as an MMP inhibitor indicated only for the treatment of periodontitis while in the dermatology doxycycline is successfully used in the treatment of skin disorders of inflammatory nature, like acne and rosacea (Di Caprio et al. 2015; Korting and Schöllmann 2009). Minocycline has also been found to have beneficial effects in inflammatory conditions and is

indicated for the treatment of rheumatoid arthritis (Good and Hussey 2003).

One of the major non-antibiotic roles of tetracyclines is in mediating cytokine production from immune cells (Nikodemova et al. 2006). They interfere with cytokine production from neutrophils and macrophages during inflammatory conditions, in particular suppression of TNF-α, IL-1β, and IL-6, found following the tetracyclines treatment of inflammatory skin disorders (Sapadin and Fleischmajer 2006). Low doxycycline doses were more effective than high doses in modulating gene expression of lipopolisaccharide-induced pro-inflammatory cytokines (IL-8, TNF-α, and IL-6) in immortalized human keratinocytes (Wang et al. 2017). Minocycline has anti-inflammatory effects on neutrophils and monocytes by inhibiting neutrophil-mediated tissue injury via inhibition of neutrophil migration and degranulation, and by suppression of lipopolysaccharide and IF-γ-induced NOS expression and NO release from monocytes (Amin et al. 1996). Modulation of T cell activation and function has been proposed as one of the minocycline mechanisms of action in inflammatory conditions whose pathogenesis is significantly T cell driven. The effects of minocycline in $CD4^+$ T cells were associated with its impact on the activity of the transcription factors like NF-κB, AP-1 (activator protein 1), and NFAT (nuclear factor of activated T cells) as well as with attenuation of intracellular Ca^{2+} levels (Szeto et al. 2011).

Inhibition of microglial activation. In vitro studies demonstrated that minocycline exhibits a direct inhibition of the proliferation and the activation state of the cultured microglia; it inhibited the microglial release of inflammatory mediators and excitotoxin such as NO, TNF-α, IL-1, IL-6 (Stoilova et al. 2013; Tikka et al. 2001). Since microglial activation contributes to glutamate excitotoxicity, this minocycline-induced inhibition of microglial activation was accompanied by alleviation of excitotoxicity (Tikka et al. 2001). However, minocycline did not exert an inhibitory activity on astrogliosis as found in a model of focal brain ischemia (Yrjänheikki et al. 1999). Minocycline demonstrated anti-inflammatory and antiapoptotic effects on neurons and microglial cells in several models of brain injury like global and focal cerebral ischemia, traumatic brain injury, spinal cord injury and intracerebral hemorrhage, in which it provided both reduction in tissue injury and improvement in functional recovery (Elewa et al. 2006). Minocycline treatment was reported to reduce the incidence and severity of encephalitis in simian immunodeficiency virus macaque model of HIV-associated neurological disease by decreasing cytotoxic lymphocyte infiltration into the brain, and it reduced p38 activation in primary lymphocytes in vitro (Zink et al. 2011). Experiments in mice expressing a mutant superoxide dismutase (SOD1G37R) and mimicking human ALS, demonstrated that daily administration of minocycline (1 g/kg) into the diet, initiated at late presymptomatic stage (7 or 9 months of age), delayed the onset of motor neuron degeneration, muscle strength decline, and increased the longevity by 5 weeks for 70% of tested mice (Kriz et al. 2002). These beneficial in vivo effects of minocycline were associated with attenuated microglia activation in the brain at early symptomatic stage, and in the spinal cord at the end stage. However, another study on mice ALS model demonstrated that minocycline administration at a late stage of ALS had no effect on the animal survival and, on the contrary, may not have anti-inflammatory properties as it significantly altered glial responses and exaggerated neuroinflammation (Keller et al., 2011; Orsucci et al. 2012). Doxycycline was found to suppress microglial activation induced by lipopolysaccharide by modulation of p38 MAP kinase and NF-kB signaling pathways (Santa-Cecília et al. 2016).

Briefly, presented literature data indicates that minocycline and doxycycline have some neuroprotective properties against the ND-related pathology in vitro although the exact mechanisms in achieving them are not completely understood. Although each of their various non-antibacterial activities may contribute to the neuroprotection, focusing on only one of the mechanisms has not been proved effective so far (e.g. monotherapy with conventional drugs having only anti-inflammatory or antioxidant activity did not result in improvement, reversal or block of the ND-related pathology), due to it seems most likely that all of the above mentioned non-antibiotic activities act synergistically and complementary in achieving the desired neuroprotective effect (Stoilova et al. 2013). Considering previously mentioned common pathomechanisms of NDs, no data on tetracyclines activity on IRBS could have been found in the available literature. However, the importance and predominance of the particular mechanism should not be discussed on a general basis and could be difficult to assess as, among other factors, it probably depends on the underlying pathology of the certain ND and especially the disease stage (e.g. antiamyloidogenic activity against pre-oligomeric forms or different activity on the early versus late stage of ALS), and to that related subtle differences in the drugs' structure-activity relationship. For example, some reports suggest that both minocycline and doxycycline inhibit the aggregation pathway of Aß1-42 and decrease the accumulation of its oligomeric species and fibril formation but minocycline seems to have higher inhibitory activity (Xu et al. 2020), while in case of α-synuclein, in contrast to doxyxcyline, minocycline seems to be ineffective in vitro in inhibiting its aggregation and halting the fibrils grow (González-Lizárraga et al. 2020). Both disease/stage- and drug-dependent differences can have a further impact on the involvement of their anti-inflammatory, antioxidant and other "anti-…" neuroprotective activities as, for example in case of α-synuclein, oligomeric species elicit toxic effects by altering the membrane permeability leading to influx of calcium, mitochondrial damage etc, while fibrillar species provoke toxicity, among other mechanisms, mostly by triggering inflammatory processes (González-Lizárraga et al. 2020). It is up to the in vivo studies on animal ND models and particularly the clinical trials in patients with NDs to shed more light to this issue.

3.2. Minocycline and doxycycline therapeutic potential in Alzheimer's and Parkinson's disease

The pleiotropic biological activities of minocycline and tetracycline observed in vitro (especially the antiamyloidogenic, anti-inflammatory, antioxidant and antiapoptotic ones) need to be confirmed for their effectiveness in producing a neuroprotective response in animal ND models and preliminary clinical trials in NDs patient populations (Stoilova et al., 2013). Particular emphasis in this review has been put on animal and human studies of AD and PD as the two most common NDs.

3.2.1. Tetracyclines and Alzheimer's disease

Animal AD models.

MINOCYCLINE. There are over 200 publications on the therapeutic potential of tetracycline antibiotics (mostly minocycline) in NDs, with a majority of them referring to preclinical research. Among them, there is only one meta-analysis which provides parallel determination of the effect of minocycline monotherapy on different animal ND models (AD, PD, Huntington disease /HD/) and stroke in rodents (Li and Schluesener 2013). The meta-analysis included 19 studies from the time interval 2000 to 2012, which were mostly conducted on mice (11/19)

and less on rats (7/19). It is important to emphasize that AD models were explored in only 5/19 studies, among which 3/5 used transgenic mice implying the familial form of AD (Fan et al. 2007; Ferretti et al. 2012; Seabrook et al. 2006), while 1/5 study used non-transgenic rats treated intracerebroventricularly with an immunotoxin p75-saporin (Hunter et al. 2004) and 1/5 study used both transgenic mice and non-transgenic rat model induced by a central administration of Aβ1-42 (Choi et al. 2007).

Seventeen of these 19 studies included drug dose that was equal or higher than 45 mg/kg/day (mean value 60 mg/kg/day) and in AD animal studies it generally varied in a narrow range of 45-55 mg/kg/day (Li and Schluesener 2013). The authors have concluded that a dose of 45 mg/kg/day (which in rats and mice approximately correlates to adult human daily dose of 500 mg and 250 mg, respectively) is consider to be moderately high, and only 1/5 AD studies used a low dose of 10 mg/kg/day. In contrast to the drug dose, the age of animals at which the minocycline treatment was initiated in these 5 AD studies varied in a huge range of 7 – 48 weeks, corresponding to different disease stages, from pre-symptomatic/pre-clinical to more advanced ones. Most frequent route of drug administration in different ND models (13/19) including the AD models was intraperitoneal which is in contrast to what could be planned for a possible use in AD patients. Although in humans it is to be expected that such (oral) therapy should last several years, studies performed in AD animals from this meta-analysis were lasting not longer than 3 months. In 2/5 AD studies drug was given for 12 weeks in a chow or as subcutaneous injection, while in the other 3/5 studies treatment duration was 3-4 weeks, with mostly anti-inflammatory (4/5) and, to a lesser extent (2/5), antiapoptotic (the reduction of neuronal cell death) effects being explored. The anti-inflammatory effect was measured by drug ability to reduce the accumulation of amyloid beta plaques, also to inhibit microglial activation, and to reduce the interleukin and TNF levels. Assessment based on a reduction of the amyloid plaques accumulation has shown greater efficacy when minocycline was administered in moderate doses than when administered at a low dose. Generally, the results obtained in these 4 AD animal studies but also in studies on other models of neurodegeneration in this meta-analysis, provide a strong support to the anti-inflammatory activity of minocycline while insufficient data have been available in the meta-analysis for such a strong conclusion on its antiapoptotic effects in spite of such a tendency. The antioxidant activity of minocycline has not been explored in these studies.

Anti-inflammatory, antiamyloidogenic, antiapoptotic and antioxidant activities have been proposed to be the major determinants of the minocycline-induced positive effects on cognitive impairments in NDs, and yet, according to this meta-analysis, cognitive performance in AD animal studies was not tested in all studies. One month-treatment with minocycline (50 mg/kg i.p.), initiated in 1-year old transgenic AD mice, significantly improved their spatial learning memory performance (but not fully rescued) which was accompanied by a reduction in activated microglia and IL-6 levels without any effects on the amounts of soluble, insoluble or oligomeric Aβ, or the ratios of Aβ1-40 and Aβ1-42 (Fan et al. 2007). Study of Seabrook and coworkers (Seabrook et al. 2006) assessed the effectiveness of 3-month treatment with food containing minocycline (55 mg/kg/day) given prior to and early in Aß plaque formation in APP transgenic mice, and revealed that minocycline has different effects at different ages on Aß deposition and cognitive improvement, found affected in younger (increased Aß deposition but improved cognition) but not in older transgenic animals (no change in Aß deposition and no cognitive improvement). In a model induced by central application of an immunotoxin in mice aged 2-3

months, one month-treatment with minocycline initiated immediately after the toxin, prevented cognitive impairment by reducing the cholinergic neuronal loss (Hunter et al. 2004). Another study (not included in this meta-analysis) on double transgenic APP/PS1 mice AD model which explored the effect of minocycline treatment on microglia activation and neurogenesis, revealed that minocycline (50 mg/kg ip) given twice a day during first 2 days followed by a once daily treatment for another 5 days before the GFAP retroviral vector infusion, and continued with a daily dose of 25 mg/kg for the next 5 weeks, had no effects on brain levels of Aß and Aß-related morphological deficits although it inhibited the microglia activation accompanied by protection of hippocampal neurogenesis in mice sacrificed at 4-month of age (Biscaro et al. 2012). However, a study on transgenic mice overexpressing APP751 demonstrated that minocycline treatment (50 mg/kg/day, route of administration not specified) initiated a month prior to the appearance of extracellular amyloid plaques, resulted in lowered levels of Aß trimers associated with corrections of behavioral deficits and decreased levels of inflammatory markers (Cuello et al. 2010).

Other literature data indicates some beneficial effects of minocycline treatment also in the non-transgenic AD models. The protective effects of minocycline acute (24h; 20 mg/kg i.p.) and sub-chronic (14 days; 5 mg/kg i.p.) pre-/post-treatment were demonstrated in a non-transgenic mice AD model induced by intracerebroventricular administration of streptozotocin (STZ-icv), presented as normalization of both up-regulated neuroinflammatory genes and impaired mitochondrial function/decreased gluthatione level in the hippocampus, accompanied by improvement in depressive-like behavior (Mozafari et al. 2020). It should be mentioned that these effects were achieved by much lower doses than those used in other studies. In a non-transgenic mice AD model induced by intracerebroventricular administration of Aß1-42, high dose of minocycline (50 mg/kg/day per os) administered for 15-17 days improved the spatial memory (Garcez et al. 2017) and prevented the development of depression-like behavior (Amani et al. 2019), which in both cases was associated with reduction in inflammatory parameters.

The effects of minocycline on other AD-like hallmarks like hyperphosphorylation of tau protein or metabolic pathology like insulin brain resistance and cerebral glucose hypometabolism were not the assessed by studies reviewed by (Li et al. 2013). However, literature data outside of the meta-analysis of Li and coworkers (Li et al. 2013) demonstrated that short-term treatment with a low minocycline dose (10 mg/kg i.p. for 14 days) was capable of reducing tau aggregate load in transgenic mice (htau line) both in the early stages of neurofibrillary tangles development (3-month old animals) and in the advanced stage with mature tau pathology (12-month old mice) (Noble et al. 2009). This research pointed out that conformational changes in tau are susceptible to minocycline treatment, but are not directly associated with the amount of tau fragments produced. In their extended research this group demonstrated that in young htau mice (3-4 month of age), minocycline treatment reduced cortical but not hippocampal astrocytosis (Garwood et al. 2010). However, the research of Parachikova and coworkers, performed on 8-month 3xTg AD mice treated orally with a higher minocycline dose (55 mg/kg/day) for 4 months, indicated that although such a treatment restored cognition and reduced Aß-induced neuroinflammation, it did not have a significant effect on tau hyperphosphorylation (Parachikova et al. 2010). Inconsistent findings on minocycline effects on taupathology obtained in a research of Noble and coworkers (Noble et al. 2009) and Parachikova and coworkers (Parachikova et al. 2010) indicate that methodological (low vs high dose; i.p. vs oral administration) and model (distinct inflammatory responses and individual pro-inflammatory cytokines)

differences might be of utmost importance in interpretation and translation of the data from preclinical to clinical studies.

Side effects with minocycline have been reported rarely in animal studies, the available data indicates that minocycline may have variable and even deleterious effects in different species and models according to the route of administration and dose (Ferretti et al. 2012; Li et al. 2013).

DOXYCYCLINE. Literature data on in vivo testing of the therapeutic potential of doxycycline in animal AD models is not as extensive as the one on minocycline. Sub-chronic (20-day) and chronic (2-moth) treatment with doxycycline (10 mg/kg i.p.) in old double (APP/PS1) transgenic AD resulted in cognitive improvement but was not associated with plaque load reduction under the achieved brain concentration lower than 1 μM that were previously shown to be below the concentration threshold for antiaggregating effects observed in vitro (Balducci et al. 2018; Forloni et al. 2002). However, in the same study direct intracerebroventricular administration of doxycycline alone caused memory deficit comparable to that of mice treated intracerebroventricularly with synthetic Aß1-42 peptide, indicating a negative effect of the drug itself when directly injected into the brain (Balducci et al. 2018). The exact mechanism of this direct toxicity is unknown and no similar data are yet available for direct administration of minocycline. Administration of doxycycline to Drosophila model of AD (Aß1-42-expressing flies) slowed the progression of their locomotor deficits but did not improve the lifespan of flies (Costa et al. 2011).

Clinical AD trials. Search of the Clinicaltrials.gov database and the PubMed engine revealed only several clinical studies that investigated the efficacy of tetracyclines in patients with Alzheimer's disease.

MINOCYCLINE. The effect of minocycline was investigated in two studies. A small interventional study that enrolled 5 cognitively impaired patients (4 AD, 1 Mild Cognitive Impairment /MCI/) and 8 healthy controls investigated the effect of minocycline monotherapy in a dose of 50 mg twice daily given for 6 months (https://clinicaltrials.gov/ct2/show/NCT01463384, no date). Performance assessment for the primary outcome was done via the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) where immediate and delayed memory, attention, language, and visuospatial skills were measured. Participants with AD and MCI had scores below baseline values at the time intervals of 1–3 months and averaged for after 4–6 months during the treatment (https://clinicaltrials.gov/ct2/show/NCT01463384, no date). No side effects were reported in the study, and liver and kidney function tests (alanine transaminase and blood urea nitrogen levels) performed on a monthly basis revealed no abnormalities. Howard and coworkers investigated whether minocycline slows the decline in cognitive and functional ability in 554 participants with mild AD over a 24-month treatment period and whether a higher minocycline dose (400-mg) compared to the 200 mg used in standard practice would enhance its efficacy (Howard et al. 2020). The rationale for the minocycline therapy was its anti-inflammatory properties that have been expected to concur the neuroinflammatory toxicity in AD pathophysiology. The primary outcome variables were the Standardized Mini-Mental State Examination score (SSMSE) and the Bristol Activities of Daily Living Scale (BALDS) assessment. The results of the study showed that minocycline (regardless the dose) does not slow down the cognitive decline of people with AD and that it has no effect on improving their functioning.

There was a greater loss from the study in subjects who received minocycline at a higher dose, primarily due to gastrointestinal and dermatological side effects indicating that 400 mg dose of minocycline is poorly tolerated in AD patient population (Howard et al. 2020). The authors have offered three possible speculations on the drug failure; (i) neuroinflammation may be only a reaction to the pathology and not an important factor in neurodegeneration, (ii) minocycline might interfere with the supportive function of microglia which may reduce its beneficial effects, or (iii) the anti-inflammatory treatment effect of minocycline was too small to be detectable (Howard et al. 2020). Gyengesi and Münch provided some arguments against these explanations elaborating that (i) >60% of the genes linked to sAD are inflammation-related and that TNF-blocking agents reduce the risk of AD in patients with psoriasis and rheumathoid arthritis, (ii) minocycline has not been designed to act as anti-inflammatory drug and its mechanisms of actions are not entirely clear, it is, therefore, unknown whether the levels of relevant pro-inflammatory cytokines could have been adequately decreased by the dose used in the study, and (iii) small effect size could reflect the contribution of process other than neuroinflammation (Gyengesi and Münch 2020). To avoid these obstacles in a search for the effective anti-inflammatory drug for AD, instead of tetracyclines, they have suggested the preferable use of anti-inflammatory drugs with known targets (e.g. the cytokine-suppressive anti-inflammatory drugs) and exploiting of imaging studies of a marker of activated microglia and astroglia in the brain to monitor the anti-inflammatory activities in vivo (Gyengesi and Münch 2020).

DOXYCYCLINE. Two studies investigated the efficacy of doxycycline either as a monotherapy or in combination with rifampin. A preliminary randomized, triple blind, controlled trial by the group of Loeb conducted on 101 patients with probable AD and mild to moderate dementia who were treated orally with a combination of daily doses of doxycycline 200 mg and rifampin 300 mg for 3 months, demonstrated a significantly less decline in the SADAS-cog score at 6-month but not at 12-month time-point, respectively, while significantly less dysfunctional behavior was detected only at the 3-month time-point, compared to placebo-treated group (Loeb et al. 2004). The results encouraged the group to performed another, larger study designed as a multicenter, randomized blinded, 2×2 factorial controlled Phase-3 trial (the DARAD trial) which involved 406 subjects to examine the effect of doxycycline and rifampicin (monotherapies and combination) on the cognitive performance (variables were the scores of the Standardized Alzheimer's Disease Assessment Scale /SADAS/ and the Clinical Dementia Rating Scale-Sum of the Boxes /CDR-SB/) of individuals with mild to moderate AD (Molloy et al. 2013). The drugs were administered for 12 months in the following 4 regimens (1:1:1:1); doxycycline 100 mg twice daily + rifampin 300 mg daily or doxycycline 100 mg twice daily + placebo-rifampin daily or rifampin 300 mg daily + placebo-doxycycline twice daily or placebo-doxycycline twice daily + placebo-rifampin daily. The trial was stopped early because of futility due to the consistent results (both primary and secondary outcomes) of the interim analysis performed on 306 patients showing that none of the active treatments was significantly better than placebo and that there was less than 1% chance of attaining the treatment goals if the study was fully recruited. Weight loss and gastrointestinal side effects were more commonly reported in patients treated with the combination of drugs compared with the monotherapies or placebo while hematological adverse events were more common in participants receiving rifampicin monotherapy compared to other treatments. Although the group used the same patient population to registered studies exploring the Magnet Resonance Imaging data and laboratory analysis results on plasma/CSF pro- and anti-inflammatory cytokines and amyloid and tau levels

(Clinicaltrials.gov database), the exact results were not submitted nor published in PubMed. Therefore, since the biochemical/neurochemical parameters of oxidative stress, neuroinflammation, apoptosis and brain insulin resistance have not been measured in clinical settings, it could not be concluded whether the mechanisms of neuroprotective effects of tetracyclines seen in preclinical studies have been faithfully reproduced in the AD patients or some other mechanisms are involved with a more important influence on the clinical outcome. On the other hand, it has been speculated that since the trials were performed with patients diagnosed with AD, amyloid oligomeric species were probably already formed before the treatment with minocycline and rifampine was initiated, which, according to the in vitro studies, could account for the minocycline failure to improve the AD condition (Socias et al. 2018).

3.2.2. Tetracyclines and Parkinson's disease

Animal PD models. Most frequently exploited animal models of PD are non-transgenic models induced by peripheral administration of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP), a toxin that selectively destroys nigrostriatal dopaminergic neurons, and models induced by a central administration of 6-hydroxydopamine (6-OHDA), a hydroxylated analogue of dopamine which is a selective substrate for the membrane dopaminergic transporter (DAT) and induces oxidative stress-related cytotoxicity and mitochondrial fragmentation, or by rotenone, a naturally occurring organic pesticide that induces mitochondrial dysfunction and ultimately cell loss in the nigrostriatal pathway (Gubellini et al. 2015).

MINOCYCLINE. Beside some review articles dealing with tetracyclines' therapeutic potential in animal PD model (Kim et al. 2009; Orsucci et al. 2009) the only one meta-analysis focused on this topic is the one mentioned previously for AD models which explored the effectiveness of minocycline (Li and Schluesener 2013). Only 3 animal PD studies presenting the results of 7 experiments have been included in this meta-analysis. The three studies were conducted in different animal species by mean of different PD-inducing agents; one study in MPTP mice model (Yang et al. 2003), the other in 6-OHDA rat model (Quintero et al. 2006) and the third in lipopolysaccharide-induced rat PD model. Minocycline dose ranged from 45 -120 mg/kg given either i.p. or orally for up to 1 month, in neuroprevention or neurorescue design, depending whether minocycline was administered before or after the PD-inducing agent. The authors of the meta-analysis concluded that the high minocycline dose (90 mg/kg/day) inhibited microglial activation, whereas the moderate dose (45 mg/kg/day) reduced TNF-α in the rat models accompanied by improvement in behavioral function, without any further details (Li and Schluesener 2013). However, thorough reading of the results of these studies revealed some additional findings which seem to be very important for a proper translation of preclinical data to human studies exploring the therapeutic potential of tetracyclines in NDs. The results of Quintero and co-workers suggested that the time of minocycline delivery relative to neuropathogenic event may determine the effectiveness of this drug for therapy based on the significant reduction in loss of tyrosine hydroxylase-positive cells when minocycline therapy was initiated before 6-OHDA (neuroprevention) and a much lesser and insignificant effect when it was initiated after the 6-OHDA administration (Quintero et al. 2006). Additionally, the results of Yang and coworkers (Yang et al. 2003) actually indicated worsening of the MPTP-induced damage to the dopaminergic neurons as will be explained in more details further on in the text.

Recent research conducted by Wang and coworkers on the PD transgenic mouse model generating by use of recombinant adeno-associated virus (AAV) to overexpresses human mutant α-synuclein (A30P-A53T) in the substantia nigra, also assessed the effects of minocycline treatment (30 mg/kg once daily for 1 month, initiated after AAV administration) (Wang et al. 2020). Such a treatment was found to inhibit microglial activation by reducing the level of IL-1ß, known to be released from activated glia following AAV administration, which play an important role in the α-synuclein-mediated pathology. This beneficial effect was associated with prevention of the dopaminergic neuronal loss and increased the dopamine level in the nigrostriatal region, additionally accompanied by reversal of the behavioral dysfunction, motor discoordination and disturbed spontaneous exploratory activity, assessed 12 weeks after AAV administration (Wang et al. 2020).

Beneficial effects of minocycline treatment manifested as block of the microglial activation have been reported as well in non-transgenic PD models (Cankaya et al. 2019). The effect was seen in 6-OHDA mouse model after intraperitoneal doses of 45 mg/kg for 14 days (He et al. 2001), as well as in MPTP mouse treated with intraperitoneal doses ranging from 1.4 – 45 mg/kg initiated immediately after the first MPTP dose and continued for 4 days after the last MPTP dose (Wu et al. 2002). In this study minocycline specifically blocked microglial but did not alter astrocytic response, and it also blocked the microglial production of IL-1 and the induction of ROS- and NO- producing enzymes such as NADPH–oxidase and iNOS. In another study on MPTP model conducted by Du and coworkers minocycline treatment (60, 90, or 120 mg / kg per day in 5% sucrose) performed by oral gavage before, during, and after the MPTP administration for 9 days, prevented nigrostriatal dopaminergic neuronal loss caused by MPTP neurotoxicity, in both striatum and nucleus accumbens. Given the role of NO in MPTP neurotoxicity, reduction of iNOS as well as of caspase 1 expression with minocycline has been demonstrated (Du et al. 2001). Rotenone-induced PD model has been also explored for assessing the neuroprotective effects of minocycline. Treatment with minocycline (30 mg/kg i.p.) given before rotenone administration was capable of enhancing (to some extent) the motor ability in a rotenone-induced rat PD model as manifested by increased number of ambulation and rearing and shortening of the immobility time while no effects were observed in the minocycyline-treated control rats (Sun et al. 2019). The alleviation of motor deficits was accompanied by inhibition of ROS and NO production as well as by increased tyrosine hydroxylase and Nurr expression, and the upregulated phosphorylation levels of CREB in the substantia nigra (Sun et al. 2019).

In contrast to these encouraging animal studies, some studies have showed a detrimental effect of the minocycline administered in animal PD models. Yang and coworkers (Yang et al. 2003) conducted 4 studies in MPTP mice model with minocycline treatment in doses ranging from 22.5 – 60 mg/kg i.p. (at 12-hr intervals, given 1 day before and continued 1 day after the MPTP administration) or orally in two experiments, with minocycline doses of 90 – 120 mg/kg/day given by gauge 2 days before and continued 1 day after the MPTP administration, and dose of 10 mg/kg (4x daily at 2-hr intervals) given with diet for 2 weeks before and during MPTP administration. Although these studies clearly demonstrate the ability of minocycline to block inflammatory responses resulting from MPTP treatment, all four studies demonstrated that minocycline treatment led to a consistent potentiation of MPTP-induced striatal damage and a neuroprotective effect of minocycline have not been achieved despite use of different minocycline doses and both oral and parenteral route of its administration. Concomitant use of minocycline (as well as doxycycline or tetracycline) and MPTP caused greater loss of striatal dopaminergic

neurons and tyrosine hydroxylase activity than the use of MPTP alone. The authors demonstrated that this harmful effect was caused by acute minocycline interference with the function of vesicular monoaminergic transporter-2 (VMAT2) to mediate the uptake of 1-methyl-4-phenylpridium (MPP+), thus causing perturbation in intracellular sequestration of MPP+ which resulted in increased concentration of free MPP+ and its mitochondrial accumulation leading to more extensive damage to dopaminergic neurons (Yang et al. 2003). This research revealed a novel mechanism of action of minocycline which, bearing in mind the importance of VMAT2 functioning for dopamine uptake and normal dopaminergic neurotransmission, should be considered when exploring minocycline as a promising neuroprotective agent for the treatment of NDs, and PD in particular. Similar findings of harmful minocycline effects were reported in a study conducted by Diguet and coworkers which examined the effect of minocycline on the MPTP female monkey model following 15-day treatment with a dose of 200 mg/kg twice daily (Diguet et al. 2004). Monkeys who received placebo + MPTP developed symptoms of mild parkinsonism on day 15, while the group that received minocycline in addition to MPTP developed the symptoms more rapidly and severely with a more significant loss of putaminal dopaminergic nerve endings found at the autopsy (Diguet et al. 2004).

DOXYCYCLINE. Doxycycline effectiveness seems to be more explored in animal PD models than in AD models yet less than the one of minocycline. Oral (chow) treatment of 6-OHDA-induced mouse PD model with doxycycline in a dose of 40 mg/kg for 30 days decreased both astrocyte and microglia response to 6-OHDA in the globus pallidus and substantia nigra pars compacta as well the astrocyte reaction in the striatum (Lazzarini et al. 2013). These findings are in contrast to minocycline ineffectiveness on astrocytes found in MPTP mouse model (Wu et al. 2002) which may indicate a difference in the targets between these two tetracycline drugs and/or additionally, the effects of these drugs in in vitro conditions may not be entirely identical to their effects in in vivo animal models generated by different neurotoxins. Considering the targets, some literature data suggests that doxycycline interferes with α-synuclein protein by inhibiting its self-association into fibrils and forming globular rather that fibrillary aggregates, and since it binds only to aggregated species of alpha-synuclein, it would not interfere with the physiological function of monomeric alpha-synuclein (Avila et al. 2017). Recent study of Bortolanza and coworkers on 6-OHDA rat model of PD treated with L-DOPA, demonstrated that acute doxycycline treatment (40 mg/kg ip given 30 min before L-DOPA) attenuated L-dopa-induced dyskinesia while co-administration of doxycycline with L-DOPA prevented the development of dyskinesia, without modifying its anti-kinetic action (Bortolanza et al. 2021). The authors concluded that the effect is probably mediated by doxycycline-induced inhibition of neuroinflammatory processes involving glial cells, attenuation in the ROS production and metalloproteinase-2/-9 activity, metalloproteinas-3 expression. In the experiment conducted on the *C. elegans* model of PD grown in liquid media in presence of doxycycline (15 μM during 3 days), the treatment resulted in modification of the cellular distribution of α-synuclein in dopaminergic neurons with a significant decrease (55%) of the surface area of the α-synuclein distribution signal relative to the neuronal soma, suggesting the formation of larger non-toxic aggregates, that was accompanied by restored locomotor behavior (Dominguez-Meijide et al. 2021).

Clinical PD trials.

MINOCYCLINE. In contrast to the animal studies, the efficacy of minocycline in altering the course of Parkinson's disease has been investigated in a few clinical trials, in general in comparison with the efficacy of treatment with creatine, a compound used in sport activities which acts at the level of mitochondria and cellular energy production (NINDS NET-PD Investigators 2006).

The first such study was a randomized, double-blind, phase II futility clinical trial in 200 patients diagnosed of PD within 5 years who did not require drug therapy for the management of symptoms and were randomized (1:1:1) to receive creatine 10 g/day, minocycline 200 mg/day, or matching placebo for 12 months (NINDS NET-PD Investigators 2006). This study indicated that minocycline could be further investigated in phase 3 clinical trials in PD patient population in which it demonstrated a 77% tolerability. An additional 6 months of follow-up of the same patient population demonstrated that by 18 months, symptomatic treatment of PD symptoms was required in 62% of minocycline, 61% of creatine, and 60% of placebo-treated subjects, with prematurely discontinuation of 23%, 9% and 6% of participants, respectively, suggesting a concern regarding the decreased tolerability of minocycline (NINDS NET-PD Investigators 2008).

A large prospective, placebo-controlled, randomized double-blind multicenter clinical trial investigated the efficacy of oral minocycline as a drug treatment in patients with Multiple-System-Atrophy Parkinson-type (MSA-P) (Dodel et al. 2010). Patients were randomly assigned to receive 2 × 50 mg minocycline (N=32) or the matching placebo (N=31) twice daily for a period of 48 weeks. The change in the value of the motor score of the Unified Multiple-System-Atrophy Rating-Scale (UMSARSII) from baseline to 48 weeks (progression rate) was measured as the primary outcome variable while subscores and individual Parkinsonian symptoms (UMSARS) as well as the Unified-Parkinson's-Disease Rating-Scale (UPDRS) were used as secondary outcome variables. The study failed to show any beneficial effect of minocycline, either symptomatic or neuroprotective as minocycline treatment failed to improve global ratings of motor function for both primary (UMSARSII) and secondary (UPDRSIII) outcome variables over the 48-week observation period. The preliminary PET-data conducted in a small population of these patients suggested that minocycline may interfere with microglial activation. However, more than half of the patients (n = 33) withdrew from the study (72% of the minocycline group vs. 32% of the placebo group), rising further concern on the tolerability of minocycline (at least in this dosing regimen) in this patient population.

A Danish cohort study which reported the increased incidence of PD in patients with ocular rosacea, also found a reduced risk of PD in rosacea-diagnosed patients treated with tetracyclines (no data on the specification of the type of the tetracycline drugs used) (Egeberg et al. 2016). Since tetracyclines have been used in the treatment of rosacea for decades due to their MMP-inhibiting activity in endothelial cells and keratinocytes, it has been speculated that the MMP inhibition could be also responsible for their neuroprotective effects in rosacea-diagnosed patients with PD.

Similar to the clinical AD trials, parameters measured in the brain of tetracycline-treated animal PD models as indicators of the neuroprotective activity are not available for measurement in patients, so no clear conclusion can be reached on the successfulness of translational of the PD animal-to-PD patient data considering the underlying

mechanisms of the neuroprotective activity in the brain tissue. However, recent review of Forloni and coworkers has pointed to the specific anti-inflammatory approach that should be used in the treatment of PD based on the different inflammatory reactions that occur in the course of the disease both centrally and peripherally, and in which doxycycline might be considered as a potential candidate (Forloni et al. 2021). The central ones are associated with neuronal degeneration on one side, and glial alteration in the early phase of the disease on the other side, followed by reduced activation of astrocytes due to chronic inflammation and exposure to detrimental α-synuclein oligomers (Forloni et al. 2021). Additionally, there is evidence of peripheral inflammation in PD which can amplify the neuronal dysfunction already induced by known neuropathological PD hallmarks (Forloni et al. 2021).

3.2.3. Clinical effectiveness of doxycycline and minocycline in other related neurodegenerative disorders

Creutzfeldt-Jakob Disease

DOXYCYCLINE. Positive results and anti-prion activity of tetracyclines in vitro and in animal CJD models initiated clinical trials in CJD patient population. The first one was a multicenter, randomized, double-blind, placebo-controlled study which recruited 121 patients diagnosed with definitive or probable sporadic or genetic forms of CJD and disease duration of 6 months (mostly) or less, who were assigned to un oral treatment with doxycycline (100 mg/day) or placebo until death (Haïk et al. 2014). The trial was stopped for futility since the first interim analysis failed to show superiority of doxycycline over placebo in drug efficacy on prolonging the survival time. Subsequently, it has been postulated that the inclusion of patients mainly in advanced stage of disease might have been a reason for the negative result. The second randomized, double-blind phase II study included only CJD patients at an early stage of the disease who could be neuropsychologically tested before and during the trial, that were further allocated to oral treatment with doxycycline (100 mg/day, N=7) or placebo (N=6) and were visited every 2 months during the first 6 months, and then every 3 months (Varges et al. 2017). In parallel, the same group of researchers conducted an observational study on CJD patients who received a compassionate treatment with doxycycline (100 mg/day, N=55) or placebo (N=33), so the results of both studies were also combined by means of a random-effects meta-analysis (Varges et al. 2017). The primary outcome measure was survival time, with quality of life as a secondary outcome measure and none of the outcomes showed positive effect of doxycycline in a separate analysis of the randomized double-blind trial while in the observational study, survival time was found significantly increased in the drug-treated group (the marked difference between the genotype suggested a genotype-specific effect of the treatment). Combined analysis of both studies demonstrated statistically significant superiority of doxycycline treatment (p=0.049) (Varges et al. 2017) thus demonstrating some encouraging progress in this filed and paving the way for a carefully designed larger trial of doxycycline in the earliest stage of CJD.

Fatal familial insomnia

DOXYCYCLINE. Considering the indication of possible effectiveness of doxycycline treatment if given in a very early stage of CJD, the neuropreventive potential of this drug following the treatment initiation in the presymptomatic stage of disease was further tested in another prion disease, fatal familial insomnia (FFI), linked

to D178 mutation in the gene encoding the PrP. FFI is dominantly inherited and additionally clinically manifested by memory loss, disrupted sleep and motor abnormalities, and neuropathologically by spongiosis, marked neuronal loss and astrogliosis especially in thalamic region (Montagna et al. 2003). A preventional trial (DOXIFF), the first of this kind, was designed to test oral doxycycline treatment in 25 asymptomatic individuals (42-50 years old) that were members of FFI families and due to at risk of FFI; 10 carriers of the *PRNP* D178N/M129 mutation were allocated to a doxycycline-treated group and 15 non-carriers to a placebo group (Forloni et al. 2015; Forloni et al. 2019). Doxycycline has been given in a single daily dose 100 mg (200 mg since June 2019). Blood analysis and medical evaluation are performed every 6 months, and neurological and MR/MRI analyses have a follow-up of 2 years (Forloni et al. 2019). The study started in 2012 and specified an observational period of 10 years with doxycycline treatment considered effective if no more than three affected cases arise during the observational period and the study is still in progress (Forloni et al. 2019). Meanwhile, recent experiments on transgenic Tg(FFI-26) mouse model of FFI demonstrated that doxycycline treatment (10 mg/kg/day for 20 weeks) initiated at presymptomatic disease stage had some beneficial effects but did not modify the natural course of the experimental FFI disease overall (Lavigna et al. 2021). In this animal FFI model presymptomatic doxycycline treatment reduced hippocampal microglial activation and rescued cognitive impairment and motor correlates of sleep dysfunction but it did not prevent the onset and progression of motor dysfunction, clinical signs and progression to the fatal outcome, neither it changed the amount of aggregated and protease-resistant PrP (Lavigna et al. 2021).

Huntington disease

MINOCYCLINE. A small open-label study conducted on 14 patients with genetically confirmed HD explored the effect of minocycline (100 mg/day) evaluated neurologically and neuropsychologically after 6 months and 2 years of treatment for the neurological outcome (Bonelli et al. 2004). Minocycline was well tolerated and the results demonstrated stabilization of motor function at endpoint after improving in the first 6 months, with a tendency towards chorea improvement; out of 11 patients, 6 improved, 2 deteriorated and 3 remained on the same level in their total motor score (Bonelli et al. 2004). A larger phase 3 randomized, double-blind, placebo-controlled clinical trial explore the futility of the 18 months of minocycline (200 mg/day) treatment in 87 patients with HD compared to 27 placebo-treated patients with HD and came to a conclusion that it would be futile to proceed with another trial of minocycline 200 mg/day involving more participants for longer period of observation (Huntington Study Group DOMINO Investigators 2010) .

Amyotrophic lateral sclerosis

MINOCYCLINE. A multicentre, randomised placebo-controlled phase 3 trial included 412 patients with ALS for the assessment of the therapeutic effects of minocycline in escalating doses of up to 400 mg/day for 9 months (Gordon et al. 2007). The primary outcome measure was the difference in rate of change in the revised ALS functional rating scale. Since the primary outcome measure score demonstrated faster deterioration in the minocycline than in the placebo group, respectively (−1·30 *vs* −1·04 units/month; p=0·005), and the other disease-related symptoms and mortality showed tendency of worsening, it was concluded that minocycline has a harmful effect on patients with ALS (Gordon et al. 2007). Considering the safety issues, minocycline treatment (in

comparison to placebo) was associated with a higher incidence of non-serious gastrointestinal and neurological adverse events but they were not significantly related to the decline in the primary outcome score.

Considering the NDs other than AD and PD, no trial data is available for the efficacy of minocycline in patients with CJD and FFI prion diseases, and doxycycline in patients with HD and ALS, respectively.

4. Potential risks of a wide and long-term use of tetracyclines in the treatment of Alzheimer's and Parkinson's disease

The tolerability of minocycline and doxycycline in animal AD and PD models has been reported rarely in the literature, and their preclinical safety profile has been claimed acceptable generally based on their use for other indications in human medicine which, in general, have not been meant for a long-standing therapy on a daily basis. Additionally, some of the animal studies explored the non-oral route of tetracycline administration and/or dosing regimens (converted doses lower than those used in humans, and lack of data/experiments on the treatment duration > 3 months) not fully translational to the human use, which could have masked the type and/or real incidence of the adverse effects. Consequently, the clinical trials investigating doxycycline and minocycline therapy in AD and PD patients revealed variable tolerability of these antibiotic drugs and sometimes even safety concerns.

The safety issues of doxycycline and minocycline therapy in AD/PD indication should be considered at different levels; (i) the adverse effects manifested in the AD/PD patients during and after the treatment, particularly those that have not been considered in the reported clinical trials, (ii) those which may pose a world-wide threat due to a development of bacterial resistance to these antibiotics considering their use for the most common NDs whose incidence is exponentially increasing, and (iii) other consequences of long-term tetracyclines use. The clinical trials have been focused generally to the phenomenological listing of adverse effects observed in patients during the 6-12-month treatment with less attention being paid to their more far-reaching consequences (e.g. the impact of antibiotic-induced alteration in gut microbiota). When evaluating the safety profile of tetracycline use in the AD and PD condition, one should also keep in mind the pathophysiological background of these neurodegenerative disorders both peripherally and centrally and a fact that they are incurable diseases which implies daily administered long-lasting antibiotic therapy. All together should be considered and weighed when evaluating the potential risks of a wide use of these tetracyclines as a therapy in AD and PD patients.

Impact on the gut microbiota-brain axis. Recent literature data suggests that changes in intestinal flora may affect the activity of the brain by producing of various neurotransmitter and mediators as well as toxic substances and/or releasing of proinflammatory cytokines and other innate immune activators, which can either cross the blood brain barrier or send a signal to the brain via the vagus nerve (Cryan et al. 2019). In line with that, it has been suggested that gut microbial dysbiosis may lead to alterations in brain functions including the memory impairment, anxiety, and other cognitive dysfunctions and was further supported by the findings of gastrointestinal symptoms and reduced gut microbial diversity in AD and PD patients with decreased probiotic species and bacteria more commonly associated with anti-inflammatory properties, and increased putative pathobionts

(Angelucci et al. 2019; Kim DS et al. 2020; Kim MS et al. 2020; Quigley 2017; Sampson et al. 2016; Sun and Shen 2018; Vendrik et al. 2020). The composition of the gut microbiota can be significantly affected by the therapy with orally administered antibiotic which has led to the conclusion that such an antibiotic agent can have both positive (protection against the harmful microorganisms in the condition of developed dysbiosis and thus a reduction of neuroinflammation) or negative (related to their broad-spectrum activities and a consequent reduction on gut microbial biodiversity) effects as seen in some animal and human AD/PD studies (Angelucci et al. 2019; Hagelmaier et al. 2020; He et al. 2020; Tran et al. 2019; Zhuang et al. 2018).

Based on the postulates that antibiotics can positively affect the intestinal flora, new clinical trials have been driven aiming to explore if antibiotic interventions in the gut microbiota can achieve the desired response, improve clinical symptoms and change concentrations of inflammatory serum markers in PD patients (https://www.clinicaltrials.gov/ct2/show/NCT03958708?term=antibiotics&cond=Parkinson+Disease&draw=2&rank=8, no date) (https://www.clinicaltrials.gov/ct2/show/record/NCT03575195?term=antibiotics&cond=Parkinson+Disease&draw=2&rank=3, no date). Furthermore, a combination of pharmacodynamic and pharmacokinetic interaction at the level of gut microbiota and drug absorbtion (i.e. eradication of Helicobacter pylori, a microorganism whose prevalence has been found increased in PD patients) (Tan et al. 2015). Eradication of H. pylori (by tripple drug therapy, amoxicillin+clarithromycin+omeprazole) in persons with PD improves the absorption and onset time of levodopa, ON duration, motor severity, and quality of life parameters and thus improves the clinical condition of patients (Liu et al. 2017; Pierantozzi et al. 2006).

However, human and animal gut microbiota is involved in modulation of the liver proteome, transcriptome, and metabolome, in particular downregulation of cytochrome P450 3A-mediated xenobiotic metabolism, and thus has a number of metabolic activities, and can modulate the pharmacokinetics (absorption and/or metabolism) of the various drugs, and by that also their therapeutic outcome as extensively reviewed by Zhang and coworkers (Zhang et al. 2018). Therefore, by reducing the gut microbial diversity, antibiotics, especially broad-spectrum ones, have a high potential for drug interactions important in elderly people often presenting with comorbidities and related polypharmacy, which is particular true for the diseases like AD where aging is a major risk factor. In a large cohort study followed by a meta-analysis investigating the impact of commonly used drugs on the composition and metabolic function of the gut microbiota, the tetracyclines showed the strongest association with the altered pathways within the category of antibiotics (Vich Vila et al. 2020). The effect of tetracyclines in this respect revealed a highly species-specific pattern with a high sensitivity of Bacteroides fragilis species and a final effect being dependent on the B. fragilis : E. coli ratio (Keerthisinghe et al. 2019). However, the exact impact of tetracycline therapy on the pharmacokinetic of other concomitantly administered drugs in AD and PD patients has not been explored yet.

On the other hand, a case-control Finish study conducted to establish a causal relationship between previous oral antibiotic use and the development of PD in the interval from 1993 to 2014, indicated that certain antibiotics, with a strong emphasis on the antianaerobic and the broad-spectrum ones, are associated with an increased risk of developing PD (Mertsalmi et al. 2020). Exposure to tetracyclines 10-15 years before the index date in that study

was found to be positively associated with PD which seems to be comparable to the proposed time lag between peripheral initiation and motor manifestation of PD (Mertsalmi et al. 2020). In addition to these gut microbiota-linked effects, antibiotics can also have direct harmful effects on the gut epithelium consequently exposing the host to other (exogenic pathogens and toxins) harmful stimuli that may possibly be linked to AD and PD pathology. In both cases tetracycline therapy in AD and PD condition might carry long-term risk of additional pathology development.

General safety of a long-term oral doxycycline therapy has been explored in several randomized controlled clinical trials in which doxycycline was used because of its both antibiotic and non-antibiotic properties. In a 52-week long study on 132 patients with bullous pemphigoid designed to test the non-inferiority of oral doxycycline (200 mg/day) used for its anti-inflammatory activities versus prednisolone, related adverse effects were one of the secondary outcomes (Chalmers et al. 2017; Williams et al. 2017). Doxycycline appeared to be safer in the long-term but the collection of treatment-related severe, life -threatening and fatal events, performed in a non-blinded manner, indicated that at week 52 their rate was 18.2%, there were 3 treatment-related death and in total 86.2% patients experienced adverse events (mostly moderate, grade 2, no organ-system specification provided) (Chalmers et al. 2017; Williams et al. 2017). In a double-blind placebo-controlled study in patients with Q fever fatigue syndrome caused by the gram-negative intracellular coccobacillus, the group of 52 patients (out of 154 patients; 52 were on placebo and 50 on cognitive-behavioral therapy) that were randomly assigned to a 26-week treatment with oral doxycycline 200 mg/day for its antibiotic effects, reported no serious adverse events while in 2 patients gastrointestinal adverse effects led to study discontinuation (Keijmel et al. 2017). Beneficial safety profile of a long-term doxycycline treatment was indicated by a prospective open-label trial enrolling 60 patients with chronic rhinosinusitis with nasal polyps among whom 30 were subjected to 100 mg/day doxycycline for 12 weeks (preceded by 200 mg dose on the first day) used because of its anti-metalloproteinase activity (Pinto Bezerra Soter et al. 2017). No patient experienced major adverse events nor withdrew from this study because of the adverse effects, and 2 patients reported minor side-effects at the beginning of the treatment with doxycycline.

Substantial evidence emerged indicating that doxycycline can be used orally as a subantimicrobial-dose doxycycline (SDD) of 20 – 40 mg/day which still retains its anti-inflammatory properties and anti-metalloproteinase activity when used in treatment of a variety of disease as reviewed elsewhere (Bienenfeld et al. 2017; Gu et al. 2012). First such studies indicated there were no detectable effects on the normal oral bacteria or on the antibiotic susceptibilities of these bacteria following the treatment with 20 mg of doxycycline given twice daily for 9 months in patients with periodontitis (Thomas et al. 1998). Additionally, in 35 patients with periodontitis, 9-month doxycycline treatment applied in a same dosing regimen, no shift in the normal faecal or vaginal flora was observed, and there was no increase in the number of doxycycline-resistant bacteria or development of multi-antibiotic resistance (Walker et al. 2005). Since this small study is actually the only long-term trial with SDD that has been focused more extensively to assess the alterations of faecal/vaginal flora, larger trials are needed to confirm these results in NDs patient keeping in mind all specificities in gut microbiota found in these populations.

Development of bacterial resistance and its global impact. Tetracyclines remain in clinical use for the treatment of uncomplicated respiratory, urogenital, gastrointestinal, and other rare and serious infections; however, tetracycline resistance in many groups of medically important bacteria has narrowed their utility, resulting in discontinuation of use against several bacterial infections or requiring confirmation of susceptibility before use. Undoubtedly the use of tetracyclines in clinical practice has been responsible for the selection of resistant organisms which has been an important factor in the declining use of these antibiotics (Chopra et al. 1992). Furthermore, one of the biggest issues to emerge in recent years concerns the use of tetracyclines (oxytetracycline, tetracycline, chlortetracycline and doxycycline) as animal growth promoters and the implications of this practice for human health. Tetracycline antibiotics were ranked the number one class of antimicrobials used in animals between 2010 and 2015, accounting for 48% of the global consumption (OIE. Annual report on the use of antimicrobial agents in animals. Better understanding of the global situation 2021.no date).

In surveillance studies, the prevalence of tetracycline resistance in selected European countries was found to be 66.9% and 44.9% for extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and Klebsiella species (spp.), respectively (Jones et al. 2014), and global tetracycline-resistance percentages were 8.7% and 24.3% for methicillin-resistant Staphylococcus aureus (MRSA) and Streptococcus pneumoniae, respectively (Mendes et al. 2015). Tetracyclines are currently not recommended for the treatment of N. gonorrhoeae infections as monotherapy due to a significant resistance (Młynarczyk-Bonikowska et al. 2020). Resistance has not yet become a problem for most situations where tetracyclines are still the drugs of choice, e.g., treatment of infection caused by Brucella melitensis, Borrelia burgdorferi, Coxiella burnetii, Rickettsia, periodontal bacteria, Helicobacter pylori, Mycoplasma pneumoniae and possibly Chlamydia trachomatis. However, due to increased use of tetracyclines as first-line agents for acne, resistance rates as high as 25% have been reported for cutaneous propionibacteria, attributed to mutations rather than acquisition of resistance genes (Ross et al. 1998)

Resistance to tetracyclines is usually due to one or more of the following: the acquisition of mobile genetic elements carrying tetracycline-specific resistance genes, mutations within the ribosomal binding site, and/or chromosomal mutations leading to increased expression of intrinsic resistance mechanisms (Grossman 2016). Three tetracyclines class-specific mechanisms have been described; efflux, ribosomal protection, and enzymatic inactivation of tetracycline drugs. All of these mechanisms reduce the efficacy of tetracyclines, calling for increased rigour when clinicians prescribe these drugs (Ian and Marilyn 2001; Yılmaz and Özcenzig 2017). Tetracycline resistance develops most often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a cytoplasmic protein that interacts with the ribosomes and allows the ribosomes to proceed with protein synthesis even in the presence of high intracellular levels of the drug (Roberts 2003).

Tetracycline resistance increased rapidly in many bacterial species as a result of horizontal exchange of resistance genes on mobile genetic elements such as plasmids and transposons. A number of tetracycline resistance genes commonly found on transposable elements are often linked together with determinants for resistance to chloramphenicol or macrolides in Gram-positive organisms (Thaker et al. 2010). Efflux resistance genes are generally found on plasmids, whereas genes involved in ribosome protection have been found on both plasmids and self-transmissible chromosomal elements (conjugative transposons). Conjugative plasmids have significantly

contributed to the spread of efflux genes within the gram-negative and the gram-positive bacteria (Speer et al. 1992).

Only a limited number of bacteria acquire resistance by mutations leading to increased expression of intrinsic resistance mechanisms, such as altered permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, changed regulation of innate efflux systems, or altered 16S rRNA (Ian and Marilyn 2001). Resistance in one of the drugs in this class, typically means resistance to all tetracyclines. However, there are differences in resistance among species of bacteria.

Tetracycline-resistance has been partially countered by chemical modifications of earlier natural product derivatives and development of the semisynthetic second-generation tetracyclines, such as minocycline and doxycycline, and the latest generation of semisynthetic tetracyclines such as tigecycline, sarecycline and omadacycline. More recently, a fully synthetic tetracycline has been discovered, eravacycline, which shows promise in the treatment of serious infections caused by a broad range of bacterial pathogens. The newer agents exhibit broad-spectrum antibacterial activity similar to the tetracyclines but with improved potency against difficult-to-treat emerging multidrug-resistant (MDR) Gram-negative and -positive pathogens, including bacteria with tetracycline-specific resistance mechanisms. Tigecycline, omadacycline and eravacycline are not affected by the two major mechanisms of tetracycline resistance: ribosomal protection proteins and many efflux pumps (Zhanel et al. 2004).

Even if older tetracyclines become obsolete as clinically useful antibiotics, tetracycline resistance transfer elements will continue to be a cause for concern because they can carry other resistance genes and confer resistance to other antibiotics (Thaker et al. 2010). As with other antibiotics, increased resistance to newer tetracyclines can be expected in the future with their increased use. Furthermore, the recent discovery of a gene encoding inactivation of all tetracycline generations in nosocomial pathogens including MDR Enterobacteriaceae, Acinetobacter spp and Pseudomonas aeruginosa foreshadowed the increasing clinical resistance to latest-generation tetracycline antibiotics (Gasparrini et al. 2020; Sun et al. 2019).

Other consequences of a long-term tetracyclines use. Observational studies have examined certain complications associated with long-term antibiotic use that may not be directly related to bacterial resistance. Consequences of long term tetracyclines use have been explored mostly in patients treated with tetracyclines for acne which represent a unique and natural population in which to study the effects of long-term (>6 weeks) antibiotic use. Although these patients are much younger than patients with NDs, most of the observed side effects can be expected in the elderly patient population as well (Garrett and Margolis 2012).

Despite being uncommon, it is important for the health care provider and patient to realize that long-term use of antibiotics has been associated with important adverse events such as higher incidence of upper respiratory tract infections, collagen vascular diseases, inflammatory bowel disease and possibly increased risk for malignancy.

Infections. Patients on long-term antibiotic treatment for acne seem to have a higher incidence of upper respiratory tract infections. A study has demonstrated that nearly 35% of the patients with acne who were receiving antibiotic therapy and who had no URTI (upper respiratory tract infection) symptoms had group A streptococci in their upper airway and that nearly 85% of these strains were resistant to tetracyclines (Levy et al. 2003). The same study group investigated if the long-term use of antibiotics for the treatment of acne results in an increase in either of 2

common infectious illnesses; URTIs or urinary tract infections. They found that patients receiving antibiotic treatment for acne were more likely to develop a URTI than those with acne who were not receiving antibiotic therapy. Specifically, the odds of an upper respiratory tract infection developing in an individual with acne receiving antibiotics was 2.15 times greater than in one who was not receiving antibiotics ($P < 0.001$) (Margolis et al. 2005). This association was confirmed by a study that utilized both a cross-sectional design and a prospective cohort design. In these studies of college students, once again it was noticed that those exposed to antibiotics for acne were three times more likely to report upper respiratory tract infections (Margolis et al. 2012). Explanations for this finding include a change in the cutaneous flora distal from site of application and change in nasal colonization for those who are receiving both topical and oral antibiotics.

Although all of these studies consistently demonstrate an association between the use of antibiotics to treat acne and upper respiratory tract infections, the causality is still unclear. The diagnosis of pharyngitis is somewhat subjective, and it is possible that those with more severe acne (and more likely to receive antibiotics) are more likely to seek medical care for symptoms associated with the diagnosis of pharyngitis. In addition, the studies were not able to link a causative organism with the onset of pharyngitis.

Minocycline-induced lupus. Drug-induced lupus has been reported with tetracyclines use, although its pathogenesis remains poorly understood. Research data indicate that the rate of disease differs by gender and by drug exposure. Drug-induced lupus among those with acne is more common among users of minocycline and in young females (Schoonen et al. 2010; Sturkenboom et al. 1999). Autoantibodies associated with minocycline-induced lupus are ANA and P-ANCA. Up to 50% of patients with minocycline-induced lupus have abnormal liver function[i]. In most patients, symptoms will resolve with drug discontinuation. Resolution usually occurs between 1 and 7 months after stopping therapy; in most cases within 2 to 3 months after discontinuation (Schlienger et al. 2003).

Studies have found that the risk of developing lupus was greater after a total of 300 days of use or exposure to more than 50,000 mg of the drug, and that it often required treatment (Margolis et al. 2007).

Inflammatory bowel disease. One retrospective cohort study found an association of inflammatory bowel disease (IBD) with doxycycline use (adjusted hazard ratio [HR] =1.58; 95% CI, 1.02–2.46) and a possible association with tetracycline antibiotic use in the development of IBD in patients with acne (adjusted HR = 1.39; 95% CI, 1.02–1.90) (Margolis et al. 2010). This association was greatest for Crohn's disease and doxycycline exposure. The other tetracyclines were moderately associated with Crohn's disease but not ulcerative colitis.

Malignancy. Studies have explored the association of long-term use of antibiotics for acne (clindamycin, trimethoprim, macrolides, tetracyclines) or any indication with malignancy (breast cancer, lung cancer). The biological hypothesis for the association is that antibiotics disrupt the gut microbiome, which negatively affects the synthesis of substances that protect against cancer. Also, antibiotics can alter the inflammatory milieu.

However, according to available data the causality could not be ascertained. Thus, the increased risk found in studies with use of antibiotics may be biased and in reality associated with other risk factors that might increase the likelihood of receiving an antibiotic prescription (Bartlett 2004; Friedman et al. 2006; Zhang et al. 2008).

Other adverse effects. Tetracyclines are generally safe but adverse effects can occur (Perdue and Standiford 1999). The most common adverse events are related to gastrointestinal symptoms, nausea, vomiting, or diarrhoea,

which are seen in 7% of patients taking tetracyclines. Tetracyclines also cause dizziness, headache, and photosensitivity in approximately 2% of patients. In addition, tetracyclines are associated with rare hypersensitivity reactions (pneumonitis, eosinophilic nephritis, serum sickness) and intracranial hypertension. All antibiotics have been implicated in the development of Clostridium difficile associated diarrhoea, and this does include the tetracycline class of antibiotics.

5. Conclusion

The novel antibiotic-exploiting strategy in the treatment of AD and PD has provided a potential breakthrough in the field, initiating clinical trials driven by the success of animal studies indicating a possible neuroprotective effect of particular antibiotic drugs in NDs. The results of the in vitro analysis and in vivo experiments in animal AD and PD models provided evidence on the anti-inflammatory, antioxidant and antiapoptotic activity of tetracyclines, more or less associated with cognitive improvement which lead to investigation of the clinical efficacy of minocycline and doxycycline in AD and PD patient populations (Bortolanza et al. 2018; Santa-Cecília et al. 2019; Socias et al. 2017). However, despite the effectiveness observed preclinically in general, the available results of clinical trials are inconclusive and not supportive as it would have been expected. Problems with faithful mimicking of human NDs in respective animal models should be considered beside other possible reasons of this inconsistency. Additionally, it remains to be determined whether selective activities of minocycline and doxycycline towards the particular toxic intermediates formed in the earlier stages of abnormal protein aggregation could account for the controversial results from clinical trials in patients with NDs that were often in advanced stage of disease when the potential optimal starting point for initiation of tetracycline's therapy has passed, and/or the tetracyclines' dosing regimen might be an issue (Bortolanza et al. 2018; González-Lizárraga et al. 2020; Medina et al. 2021; Santa-Cecília et al. 2019; Socias et al. 2018). Although less explored in some animal ND models, doxycycline demonstrated better clinical effectivity and safety than minocycline (Bortolanza et al. 2018). Additionally, minocycyline was found to impair the spatial memory performance in healthy individuals in a double-blind, randomized study when administered in a dose of 150 mg twice daily for 3 days (Berens et al. 2020). All together indicates that doxycycline seems to be more appropriate drug candidate than minocycline for further clinical trials. Keeping in mind the antimicrobial property-related adverse effects of tetracyclines when used as antibiotics (100-400 mg/day), and the years-long need for the therapy of widespread NDs like AD and PD, literature data indicates that the long-term administration of SDD (20-40 mg/day) used generally to treat periodontitis or chronic inflammatory diseases with no clinical antibiotic side-effects observed so far but with some neuroprotective activities demonstrated in preclinical studies (Di Caprio et al. 2015; González-Lizárraga et al. 2017; Walker et al. 2005), should be considered for future NDs research (Bortolanza et al. 2018; González-Lizárraga et al. 2020; Gu et al. 2012; Medina et al. 2021; Santa-Cecília et al. 2019; Socias et al. 2018). This hypothesis needs a confirmation in clinical settings and further carefully planned long-term clinical trials that could provide some evidence to the clinical efficacy of such low doxycycline doses as well as the extensive drug safety profile in patients with different NDs taking into account the issues discussed above. However, it should not be neglected that NDs target the geriatric patient population in which the serum concentration of doxycycline is about twice the concentration found in middle-aged patients or healthy subjects (Böcker et al. 1986).

Therefore, further research is needed to clarify the therapeutic potential of doxycycline and minocycline in AD and PD patients but before that it would be of utmost importance to perform a thorough cost-benefit analysis paying particular attention to the long-term undesirable effects of such a long-term therapy with tetracyclines as some of them might not only lead to additional comorbidities in the patients themselves but may also pose a threat to wider patient populations. It should be kept in mind that in the treatment involving drugs possessing the antibiotic properties (especially in oral antibiotic therapy), general goal is to limit the duration of antibiotic administration to the shortest time which provides a certain clinical response, which is opposite to the needs of the years-long antibiotic therapy of NDs lacking other sufficiently effective treatments, should it be considered in this indication.

Fig. 1 Possible structure-activity relationship-based avoiding of the tetracyclines' antibacterial side-effects in their use for the treatment of neurodegenerative disorders

Considering the antibacterial activity-related far-reaching undesirable effects both for the patients and the population in general, further research on repurposing doxycycline and minocycline for a long-standing therapy of AD and PD should take into account development of the chemically modified tetracycline compounds (CMTs). Such CMTs should lack antimicrobial but retain (or introduce) those multi-targeting activities (Fig. 1) that would be most effective against the specific pathological substrates in these NDs (Bortolanza et al. 2018; Kraus et al. 2005). The research on the effects of CMTs against the neurodegenerative pathology is still modest. Recent in vitro study with a CMT-3 compound called COL-3 (CMT-3,6-demethyl-6-deoxy-4-de [dimethylamino-tetracycline]) which lacks antibiotic but poses anti-inflammatory property, indicated that it inhibited α-synuclein amyloid aggregation and led to the formation of non-toxic molecular species, and additionally disassembled preformed α-synuclein amyloid fibrils into smaller non-toxic and less inflammagenic fragments (González-Lizárraga et al. 2020). In vivo study in a rat 6-OHDA model of PD reported that CMT-3/COL-3 attenuated L-DOPA-induced dyskinesia in a rat 6-OHDA model of PD without affecting the improved motor response after L-DOPA treatment (Bortolanza et al. 2021). Tailoring of drug molecules to obtain the desired activity by means of modification of the certain regions within its structure is a well-known strategy in the modern pharmaceutical technology. Long-standing treatment with such CMT compounds would be expected to reduce the risk of the adverse effects in the gastrointestinal tract, particularly those linked to alterations in gut microbiota, but most importantly, would not contribute to development of bacterial resistance toward the tetracycline antibiotic agents. It is up to the future research to provide evidence whether such CMT compounds lacking antimicrobial effects would have enough clinical efficacy in patients with NDs while remaining a desirable safety profile, or chemical modifications of CMT molecules should consider additional structure-activity interventions, e.g. strengthening of the present non-antibiotic activities and/or even introducing the new properties to the molecules considering the current trend of the multifactorial approach to the treatment of the diversity in ND pathophysiology.

Conflict of interest. There is no conflict of interest.

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Statements and Declarations

- The submitted manuscript has not been published before and it is not under consideration for publication anywhere else.
- The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author contributions

Iva Markulin and Marija Matasin performed the literature search and participated in writing; Victoria Erdeljic Turk participated in writing and critical reading; Melita Salkovic-Petrisic had the idea for the article, drafted and critically revised the manuscript. All authors read and approved the final manuscript.

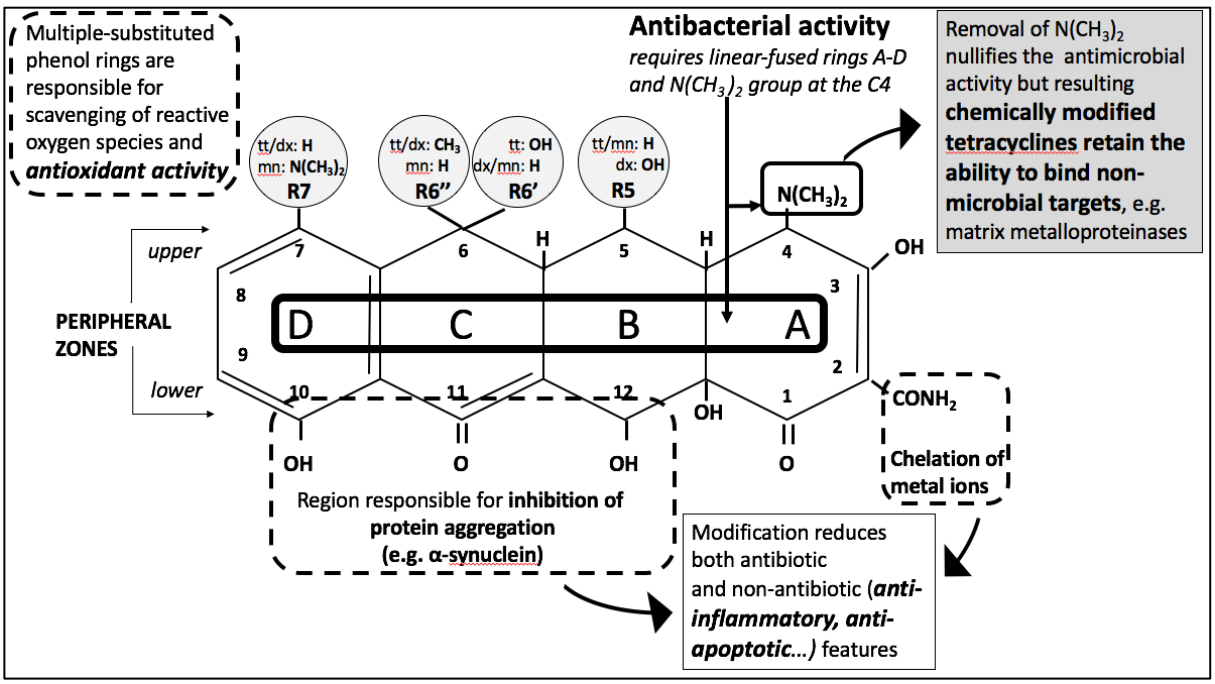

Fig. 1 Possible structure-activity relationship-based avoiding of the tetracyclines' antibacterial side-effects in their use for the treatment of neurodegenerative disorders

Chemical structure of tetracyclines reveals that different parts of the molecule are responsible for antibacterial and non-antibacterial activities. The research done so far has demonstrated that removal of the antibacterial ones preserves the ability of modified molecule to retain the non-antibacterial activities that might be useful in a struggle against the mechanisms underlying the pathophysiology of neurodegenerative disorders (Bortolanza et al. 2018; Chen et al. 2012; Stoilova et al. 2013). **tt,** tetracycline; **dx,** doxycycline; **mn,** minocycline