Lipid droplets in models of Parkinson's and stroke

Sabol, Leo

Leo Sabol

Lipid droplets in models of Parkinson's disease and stroke

DIPLOMA THESIS

Submitted to the University of Zagreb, Faculty of pharmacy and biochemistry

Zagreb, 2021

This thesis has been registered at the Pharmacology course and submitted to the University of Zagreb, Faculty of Pharmacy and Biochemistry. The research was conducted at Biomedicum Helsinki Neuroscience center unit as part of Helsinki institute for life sciences and University of Helsinki, under the expert guidance of Helike Löhelaid, PhD and Mikko Airavaara, PhD, while thesis formation was co-supervised by prof. Lidija Bach-Rojecky, PhD. Data reported in the thesis are property of the University of Helsinki, Neuroscience center, Airavaara lab.

I would like to thank all people from Airavaara lab for accepting me and guiding me through my stay with them, especially dr. sc. Airavaara for accepting me. It has been a unique experience and a true inspiration to work with you all.

Prof. Lidija Bach-Rojecky je mentorica u pravom značenju te riječi i želio bih joj se zahvaliti na svim idejama i primjerima kojima me vodila kroz zadnje godine studija, posebno na dostupnosti i pomoći kod završne faze izrade ovog rada.

Hvala obitelji i prijateljima na neupitnoj podršci i često potrebnim distrakcijama, ne bih uspio bez vas.

1. Introduction 1
1.1. The Brain 1
1.2. Ischaemic stroke 2
1.2.1. Patho- and neurophysiology 2
1.2.2. Epidemiology 5
1.2.3. Management 5
1.3. Parkinson's disease 6
1.3.1. Patho- and neurophysiology 6
1.3.2. Epidemiology 8
1.3.3. Management 9
1.4. Lipid droplets 14
1.4.1. Lipid droplets biogenesis 14
1.4.1. Lipid droplet function 17
2. Research objectives 19
3. Materials and Methods 20
Key Materials 25
4. Results and discussion 26
5. Conclussion 34
6. References 35
7. Summary 37
8. Strukturirani sažetak 38

1. Introduction

1.1. The Brain

It is impossible to see this sentence, read this sentence or understand it without brain. Due to its very complex structure, it allows people to do such a divergence of operations. Placed inside the skull, it has a beneficial position as it is close to all sense organs (Figure 1). The brain is protected by skull, vertebra from the bottom and cerebrospinal fluid in between. Alongside spinal cord, it is a part of central nervous system (CNS) with an objective, in a nutshell, is to control sensory information, integrate it and provide motoric reaction – but that can be applied to countless actions as it could be a part of regulation, processing or memory system. To make it survive, CNS collects sensory information and decides subconsciously and consciously how to preserve body's homeostasis and stay alive. Naturally, CNS also provides higher functions such us speech, creativity, emotionality, personality traits etc. For brain to function, all its parts have to be able to operate in needed way, if not – there is a possibility of a disease, increased mass of tissue, lack of some neurotransmitters etc.

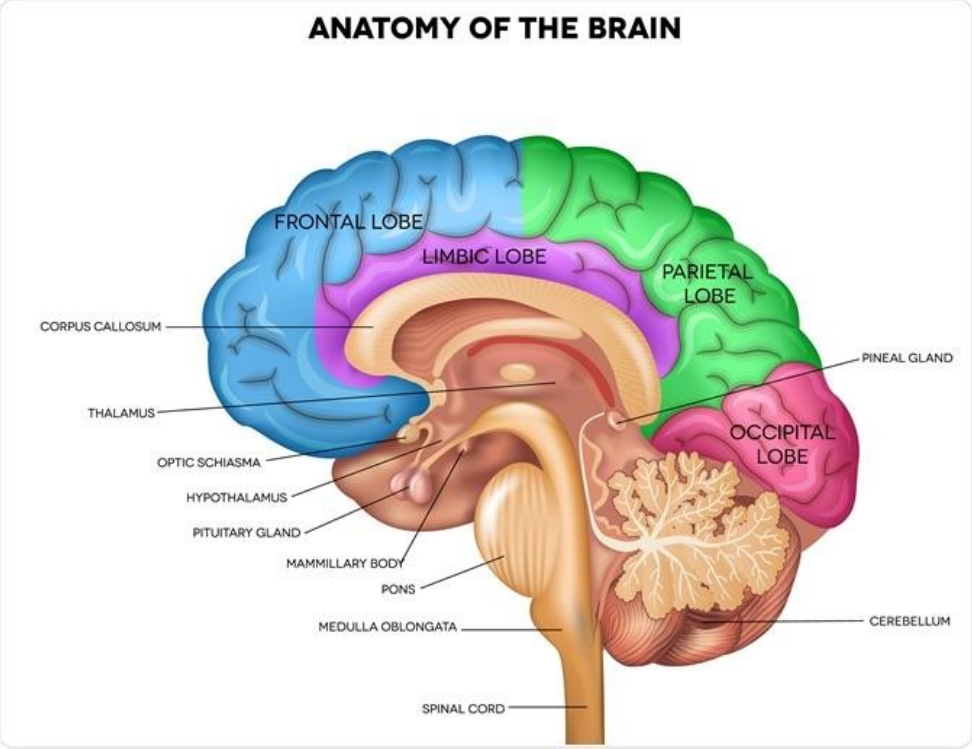


Figure 1 *Basic brain structure*

(Image Credit: https://Tefi/Shutterstock.com)

1.2. Ischaemic stroke

Stroke is defined as a neurological deficit attributed to an acute local injury of the CNS (ie, brain, retina or spinal cord) by a vascular cause.(Campbell and Khatri, 2020) However, you can divide it into 3 main types – ischaemic caused by arterial occlusion, ischaemic caused by venous occlusion and haemorrhagic (intracerebral or subarachnoid) as a result of a cerebral artery rupture (Jacobson, 2008).

1.2.1. Patho- and neurophysiology

Atherosclerosis is one of the main ischaemic causes of stroke as the artery occlusions are followed by secondary small infarcts. The most typical ischaemic stroke is caused by embolism which composes about 60% of the cases. A plaque can form in cervical arteries, in aortic arch or it can be intracranial. The third major reason is the occlusion of small penetrating vessels caused by lipohyalinosis or fibrinoid degeneration superseded by number of small infarcts – lunar syndrome. With a cerebral artery blood

flow blocked, electrical signal functionality is discontinued and therefore clinical symptoms appear. Typical brain perfusion is supplied by a flow of 60 ml/100 g tissue/min, first ischaemic symptoms are noticeable at about 22 ml/100 g tissue/min, while irreversible ischemia develops at 18 ml/100 g tissue/min. Seconds to minutes after stroke, selected regions of brain and other aerobic tissues are entering ischaemic cascade. Ischaemic cascade, a series of neurobiochemical reactions in brain is started with bioenergetic deficiency during which, because of lack of oxygen, ATP dependent ionic gradient is out of balance causing large amounts of glutamate to be called upon action and brain cells to be swelled with water. There are several consequences of that: 1) reversed access to glutamate through its membrane transporters 2) opening of the anion channels due to cell swelling 3) Ca^{2+} dependent exocytosis 4) cystine-glutamate antiporter guided glutamate exchange 5) discharge through the ionotropic purinoceptors 6) emergence of dysfunctional gap junctions due to wrongly paired connexons (Grantor, 2021). Moreover, anaerobic and hyperglycaemic conditions cause the pH to drop causing acidosis – that ends the second event in the cascade. Oxidative stress is the next event. As a result of brain ischemia, a lot of superoxide anion (O_2^-), hydrogen peroxide and later hydroxyl radical ($OH^\bullet$) precursor, is produced causing a lot of damage to the cell. Nitrous oxide (NO) is a free radical soluble in water and lipids derived from L-arginine induced by NO synthase (NOS). NOS I and III, based in nerve tissue and endothelial cells, are noticeably more active in ischaemic tissue. Meanwhile, NOS II is based glia cells and neutrophile aggregates. NOS I and II produce NO and thus damage the cell, while NOS III has somewhat protective ability, especially in the penumbra zone, causing reperfusion by vasodilation. The presence of NO also causes poly (ADP-ribose) polymerase-1, enzyme which has a part in DNA repairment, to activate. That leads to Nicotinamide adenine nucleotide (NAD^+) higher consumption and the lack of NAD^+ for some crucial bioprocesses such as anaerobic glycolysis and cell respiration. With that eventually causing ATP insufficiency, energy deficiency and nerve cell death. Brain microenvironment is protected by brain-blood barrier (BBB) – that barrier is disturbed during an ischaemic event and that is a step number 4 in the cascade. During oxidation state, matrix metalloproteinase 9 is excreted rapidly and 2 hours into the ischaemic event, basal lamina of the BBB is significantly disrupted as laminin and collagen (type I and IV) are being dissolved. The BBB disruption furthermore causes accumulation of bradykinin, vascular endothelial growth factor and thrombin. A day or

two after that, neutrophils already excreted a large number of inflammation mediators in the area, large mass molecules that transferred from vascular region into brain tissue dragged water with themselves causing vasogenic oedema which leads to intracranial hypertension and with that, secondary damage is done to the area already hit with ischemia. One of the first things to be done in acute management of stroke is reperfusion of affected area and while it has mostly positive benefits, such as lesion reduction, it can cause secondary damage (Grantor, 2021). Newly arrived leukocytes bring pro-inflammatory mediators with themselves, inducing inflammation, further BBB dysfunctionality and blood-flow cutback. Also, thrombocytes provoke a phenomenon called absence of re-flow, combined with excretion of vasoactive mediators causing spasm which induces inflammation and oxidative stress. Inside the penumbra zone, level of ischemia is considerably lower so the cells there do not experience necrosis, but rather apoptosis – programmed cell death. Ion disbalance causes mitochondrial membrane dysfunctionality which leads cytochrome C right through it. Cytochrome C, along with Bcl-2 proteins and caspases, deteriorate cytoskeleton proteins, some metabolic and signal molecules acting through endonucleases, additionally causing DNA fragmentation. While there is a lot of biomarkers known to be reliable in confirming a stroke condition, such as IL-6, CRP, TNL-α, NMDA… none has a good predictive value. Molecules like resistin and troponin are being used in diagnosing acute coronary failure which precedes ischaemic stroke. High levels of urates indicate endothelial poor function and hyperglicemia is a factor in higher death outcome prediction and lowered penumbra reperfusion chance. High cholesterol and triglycerides levels are well-known to relate to atherosclerosis, hypertension and heart failures, that being said, it would be expected for them to play a significant role in ischaemic heart stroke too. The thing is - their role is not clear. Low-density lipoprotein and triglyceride level drop after ischaemic stroke and high-density lipoproteins have a protective role in atherosclerosis-based stroke, the rest is questionable. For certain they are a part of the inflammation process, and in this thesis, they are going to be observed specifically in lipid-droplet formation and excretion (Grantor, 2021).

1.2.2. Epidemiology

The 2016 Global Burden of Disease data that were published in 2019 indicate that one in four people will have stroke in their lifetime. There are estimated to be 9,6 million ischaemic strokes and 4,1 million haemorrhagic strokes (including intracerebral and subarachnoid haemorrhage) globally each year, with a relatively stable incidence adjusted for again high-income countries but an increasing incidence in low-income and middle-income countries. (Campbell and Khatri, 2020) In 2011. in USA, health statistics present stroke as fourth leading cause of death and the primary cause of disability. While the exact timing prediction of stroke is still not known sufficiently, risk factors around which stroke is devolved on are very similar to those of cardiovascular diseases with high blood pressure leading the way in both ischaemic and haemorrhagic stroke. Other well known risk factors are smoking, hyperlipidaemia, physical inactivity, and diabetes. All of them require changes in patient's lifestyle, individual assessment and management. On the other hand, atrial fibrillation is a specific factor for ischaemic stroke that should be detected and handled promptly. Whereas medicine is still lacking in swiftness and sensitivity, there have been some major improvements. Strokes related to atrial fibrillation tend to be larger and more disabling than are strokes due to other mechanisms (Towfighi and Saver, 2011).

1.2.3. Management

A couple of practical issues that present a challenge in treating a patient with stroke; patient's age, communication ability, location of the patient – minding access from the patient to the hospital/clinical centre, health politics of the country and care-givers availability. Primary objectives should be complication reduction as pressure sores, venous thromboembolism and aspiration pneumonia could occur; starting rehabilitation as soon as possible; establishing secondary prevention. Monitoring of glucose, fever and swallowing also reduces mortality. There is a thrombus that needs to be disintegrated as it caused the stroke and it is done via intravenous administration of alteplase (currently indicated) or tenecteplase – recombinant human tissue plasminogen activators which convert plasminogen to plasmin and therefore reperfuse the ischaemic brain. Time is of the essence in treating stroke, more precisely there is a 4,5-hour window for treatment and the gain is dropping with time. Alteplase is administered in 0,9 mg/kg dose in most of the world, whereas in Japan they reduced it on 0,6 mg/kg. Tenecteplase has a longer

half-life so it is possible to administrate it a single bolus and also it's resistance to plasminogen activator inhibitors is greater. Those properties could be useful in some areas with a bad access to the patient where there would be no need for infusion on the way to the hospital as the infusion step with alteplase can represent a problem in a moving vehicle. Whilst endovascular thrombectomy has more benefit potency as a reperfusion therapy, it is reduced only on major stroke centres. Strikingly, even after successful endovascular thrombectomy, approximately half of patients with large vessel occlusion do not regain independent function. (Campbell and Khatri, 2020) Nowadays, there are some new potential targets in ischaemic stroke management, such as inhibiting postsynaptic density protein 95 to reduce glutamate excitotoxicity. For as lipid droplets, it is still to be seen what kind of an impact that would make if targeted specifically and properly (Campbell and Khatri, 2020).

1.3. Parkinson's disease

It was James Parkinson who first described Parkinson's disease or, in his words, shaking palsy: "*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace.*" Today that would be described as merely parkinsonism, not Parkinson's disease necessarily as parkinsonism concerns to a clinical syndrome incorporating tremor, rigid muscles, impaired balance, loss of automatic movements, speech changes, writing changes… Parkinson's disease would be the cause of those same symptoms that diseases like viral encephalitis or Wilson's disease can provoke. The neurodegenerative disease itself is progressive and chronic disease caused by crippling basal ganglia forcing a loss of dopamine in huge quantities (Goetz et al., 2011).

1.3.1. Patho- and neurophysiology

Primary causes of Parkinson's disease on molecular level are abnormalities in protein homeostasis and high levels of mitochondrial stress, along with genetic mutations, changes in calcium channels activity, alterations in dopamine release regulations and inflammation inside and around the neurons (Figure 2). Protein equilibrium is strictly controlled, for example by autophagy or chaperone systems. Even minor changes can

affect folding, interactions and finally, function of proteins. In terms of Parkinson's disease, maybe the most important one is α-synuclein - a protein located on the presynaptic side of cleft and found mainly in brain which can build up aggregations when folding is not successfully done. Those aggregations are called Lewies bodies. In aggregations like that, regulation mechanisms can be disrupted by separating chaperones (Heat shock protein 70 and heat shock protein 40 for example) and with that reducing their functionality. Additionally, α-synuclein aggregation may contribute to the autophagy impairments by inhibiting RAB1A protein and inhibition of the proteasome complex through chymotrypsin - like 20S/26S protein cleavage restriction. (Raza et al., 2019) Furthermore, mitochondrial complex 1 inhibitors such as rotenone and toxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) are used in preclinical studies for inducing Parkinson's-like state, for as around third of mitochondrial complex 1 cease to function in Parkinson's disease. In the disease itself, reactive oxidative species (ROS) are the main damage dealers to dopaminergic neurons originating from mitochondria. The electron leakage happens through a single electron transfer onto the oxygen molecule (O_2), producing superoxide radical ($O_2^{\bullet -}$) which can from there form hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet -}$) or peroxynitrite ($ONOO^-$) depending on the catalysing agent. In the brain affected by Parkinson's disease, dopamine is, in great measure, oxidized into dopamine quinones and free radicals. It is mostly done by monoamine oxidases (MAO-A in neurons and MAO-B in glia), but it can also be a result of self-oxidation. Oxidation products of dopamine may cyclize to form amino chromes, and are reactive enough to produce superoxide radicals. (Raza et al., 2019) When observing genetical basis of Parkinson's, there are several key genes coming into display; *Src/Park1* (mutated α-synuclein), *LRKK2, PINK1, Parkin, UCHL1, DJ-1, ATP13A2, GBA1, VPS 35.* Complexity of neurodegenerative diseases is not to be underestimated, in many of them the cause is still inseparable from the consequence. In terms CNS functionality in Parkinson's disease, it is motor cortex and basal ganglia that direct the pathway after the sequence has been started. Substantia nigra, globus pallidus, caudate nucleus and putamen together form striatum which then, combined with subthalamic nucleus, assembles basal ganglia. Striatum serves as primary input region whilst internal component of globus pallidus and substantia nigra serve as primary output regions. These two are connected via direct and indirect pathways using gamma aminobutyric acid, dopamine, and glutamate as

neurotransmitters. Essentially, direct pathway is the excitation one while indirect pathway is the inhibitory one, forcing two opposing currents into to output regions and the result is, of course, inhibition of brainstem and thalamocortical signalling which then leads to decreased motor abilities. For this thesis purpose, 6-hydroxydopamine (6-OHDA) rodent model was used to induce monoaminergic neuronal toxicity. Direct injection into substantia nigra using stereotaxic procedures is required as 6-OHDA lack blood-brain barrier permeability (Chmielarz et al., 2020).

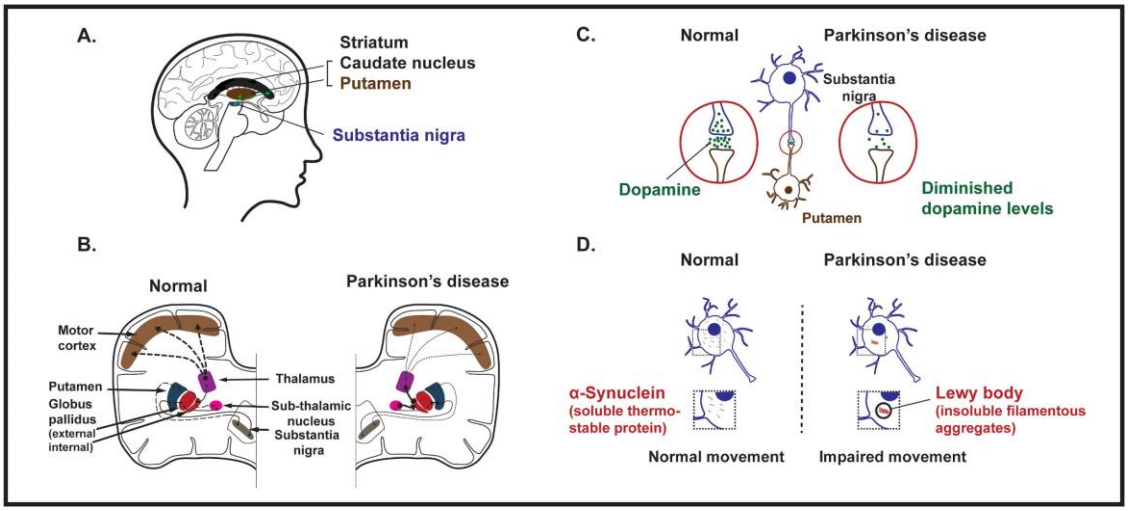


Figure 2. *The dopaminergic pathways, neurochemical and neuropathological basis of Parkinson's disease. Sketch illustrating dopamine production from Substantia nigra (blue) and synaptic transmission (green) to the striatum (A). The dopaminergic pathway with the normal target stimulation (dashed-lines) and target suppression (continuous-line) in normal brain, while degenerated Substantia nigra in Parkinson's disease impairs cortico-striatal circuits leading to induction of the associated symptoms (B). Reduced dopamine production from Substantia nigra dopaminergic neurons (C) and formation of α-synuclein aggregates as Lewy body (D) lead to the movement disorders, characterized as PD symptoms.*

(Raza et al., 2019)

1.3.2. Epidemiology

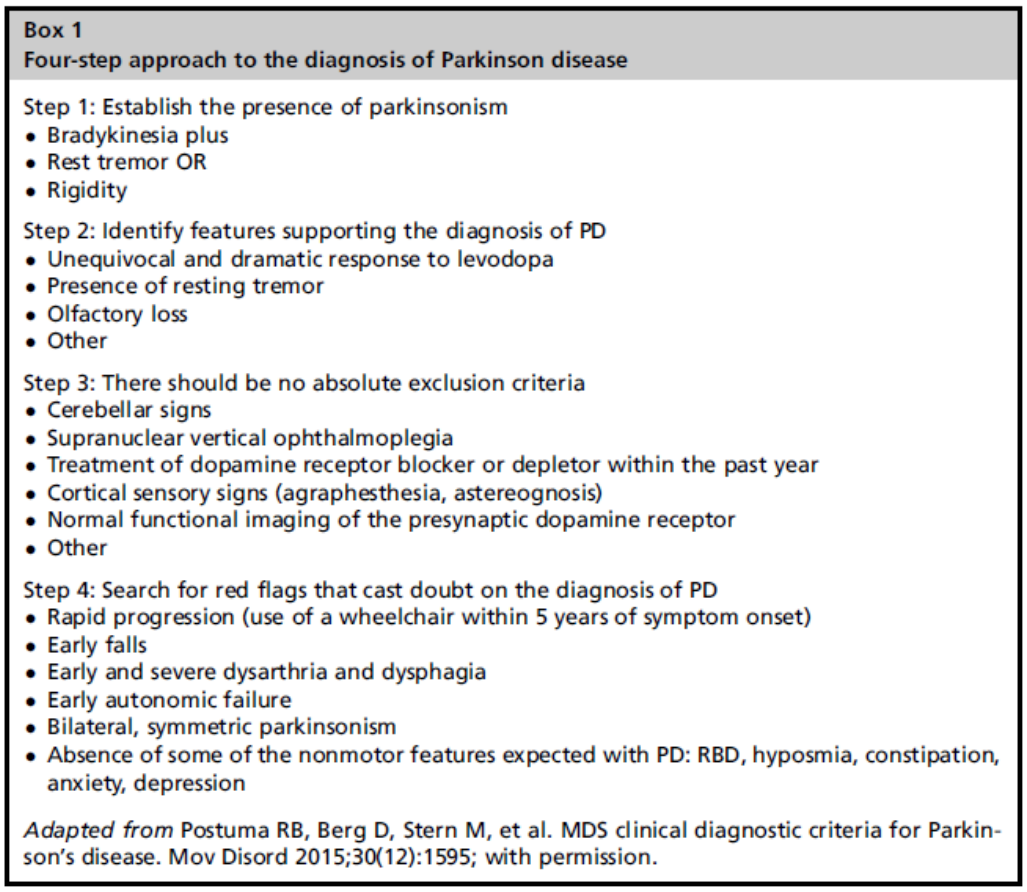
Average Parkinson's disease incidence ranges from 10 to 50/100000 persons per year whilst prevalence ranges from 100 to 300/100000 persons. While the sheer numbers are still relatively low, there is a growing trend; some studies suggest that the number of total PD cases will almost double from 2015. to 2040. It is more common in men than in women, ratio being around 1,5. Prevalence rapidly rises with age, for instance, the difference between 50-59 age group and 70-79 group is about tenfold. In studies about

geographical differences in PD prevalence, there was no significant difference between European and USA prevalence, but the prevalence in African and Asian countries was generally lower. It is important to state that there is no cure, but there are therapies which treat symptoms very well and slow the disease's progress. Even with levodopa, mortality has not dropped in such a great extent (Elbaz et al., 2015).

1.3.3. Management

Diagnosing Parkinson's disease as early as possible is extremely important as the goal is to stop neurodegeneration before too many neurons have already been lost, so steps mentioned in the table 1 should be followed. It's important to say that olfactory loss can develop years prior to any other symptoms (Figure 3).

First line of treatment are disease modifying drugs and neuroprotectors – goal is to slow down PD progression and delay use of levodopa. Selegiline and rasagiline are monoamino oxidase B inhibitors which were proven to be not effective enough. Problems are, PD pathophysiology is still not completely understood and there is a serious lack of reliable biomarkers. However, promising results are made with using α-synuclein monoclonal antibodies aiming to stop α-synuclein aggregation. Furthermore, some bright prospects are currently in development such as: active immunisation against α-synuclein, various Abelson (c-abl) kinase inhibitors such as Nilotinib and K0706, which will potentially make a breakthrough.



Box 1
Four-step approach to the diagnosis of Parkinson disease

Step 1: Establish the presence of parkinsonism
- Bradykinesia plus
- Rest tremor OR
- Rigidity

Step 2: Identify features supporting the diagnosis of PD
- Unequivocal and dramatic response to levodopa
- Presence of resting tremor
- Olfactory loss
- Other

Step 3: There should be no absolute exclusion criteria
- Cerebellar signs
- Supranuclear vertical ophthalmoplegia
- Treatment of dopamine receptor blocker or depletor within the past year
- Cortical sensory signs (agraphesthesia, astereognosis)
- Normal functional imaging of the presynaptic dopamine receptor
- Other

Step 4: Search for red flags that cast doubt on the diagnosis of PD
- Rapid progression (use of a wheelchair within 5 years of symptom onset)
- Early falls
- Early and severe dysarthria and dysphagia
- Early autonomic failure
- Bilateral, symmetric parkinsonism
- Absence of some of the nonmotor features expected with PD: RBD, hyposmia, constipation, anxiety, depression

Adapted from Postuma RB, Berg D, Stern M, et al. MDS clinical diagnostic criteria for Parkinson's disease. Mov Disord 2015;30(12):1595; with permission.

Figure 3. *Approach to Parkinson's disease diagnose*
(Reich and Savitt, 2019)

The goal of neuroprotection is to adjust neuroenvironment in a way to restore as much dopamine phenotype as possible. Several neurotrophic factors were considered to have neuroprotective properties – GDNF (glia cell derived neurotrophic factor) which effectiveness let down during clinical trials, neurturin, CDNF (cerebral dopamine neurotrophic factor) and MANF (mesencephalic astrocyte derived neurotrophic factor) where the last two show most promising results, although in less significant role as previously predicted. Few small molecules can also have neuroprotective properties such as dieadenosine tetraphosphate (AP4A) with binding sites in substantia nigra and striatum and 1,25-dihydroxyvitamin D3 (D3 vitamin) which induces GDNF. The most important drug in treating PD is levodopa, a dopamine precursor used to handle motor symptoms (Table 1). While it is very effective, there are several problems with using it: nausea and vomiting, orthostatic hypotension, sedation, confusion, sleep disturbance, hallucinations, dyskinesia and other side effects, peripheral and central metabolic conversion of levodopa into unusable substances and wearing off inside 2 years.

Therefore, it is always used in combinations to prolong the benefit. With MAOIs (safinamide), catechol-O-methyl transferase inhibitors – COMTIs (entacapone, tolcapone, opicapone) and/or dopamine decarboxylase inhibitors (carbidopa) to inhibit peripheral (and central for tolcapone) metabolism of levodopa and with that expand efficacy of the treatment. Additionally, adenosine A2 receptor antagonist istradefllyne could be added to prevent/delay off episodes characteristic for PD. Trihexyphenidyl and benztropine, anticholinergics, are antagonists of muscarine receptors postsynaptic to striatal interneurons and as such are used to weaken tremor. Although this positive impact is undeniable, these drugs are hardly used in treating PD because of their diverse side effects which include cognitive impairment, hallucinations, dry mouth, blurred vision, urinary retention and constipation. Amantadine is still a primary choice for levodopa-caused dyskinesia thanks to its potent mechanisms – antagonism of glutamate NMDA receptors, stimulating release of endogenous dopamine stores, blocking dopamine reuptake from the synaptic cleft and anticholinergic attributes. Likewise, dopamine agonists are used to delay levodopa-caused dyskinesia and to treat motor symptoms. They are separated into D2-like (D1 and D5 receptors) and D1-like (D2, D3 and D4 receptors) families where each of them stimulates different functions; for instance, D1-like stimulate memory while D2-like stimulate locomotor function. Frequently used dopamine agonists are apomorphine, ropinirole, rotigotine and pramipexole. As well as treating motor symptoms, treating non-motor ones is essential as well. Those can reduce quality of life even further. For PD-associated dementia most commonly used drugs are memantine, donepezil and rivastigmine. Typical antipsychotics can further exacerbate parkinsonism, that is why atypical ones, such as quetiapine and clozapine, are used to treat hallucinations along with pimavanserin – serotonin inverse agonist highly selective for 5-HT2A receptors. Droxidopa, midodrine, fludrocortisone and salt regulation are used to treat orthostatic hypertension while mirabegron and botulinum toxin injections are used to improve bladder dysfunction. Also, neurorestoration research has shown great progress using growth factors like CDNF and GDNF. (Airavaara, 2012) One of the most underestimated treatments is physical exercise along with speech therapy, but it can, accompanied by proper medication, severely affect disease progression. Surgical treatments can potentially have even bigger effect on PD. Ablative surgical approaches such as stereotactic destruction of physiologically defined overactive brain nuclei (thalamotomy, pallidotomy) have been largely replaced by deep brain stimulation using implanted pulse generators. The

chief advantage of deep brain stimulation over ablative lesioning is that the stimulation parameters can be customised to the needs of the patient in order to optimise the benefits. (Jankovic and Tan, 2020) For patients with severe tremor and motor symptoms most common deep brain stimulation targets are substantia nigra or globus pallidus interna. Results are yet questionable and methods are being further developed as are for cell replacement therapies and for focused ultrasound. Maybe the most promising approaches is gene therapy by CRISPR Cas gene editing or MRI-guided delivery of a viral vector, for example serotype-2 encoding the complementary DNA for aromatic L-amino acid decarboxylase into the putamen.

Table 1 Drugs used in the treatment of Parkinson's disease and levodopa-related complications

				Treatment, clinical implications and efficacy conclusions			
Class	**drug**	**daily dose range**	**Potential side effects of drug**	**To prevent/ delay disease progression**	**For symptomatic monotherapy**	**For symptomatic adjunct therapy in early or stable Pd patients**	**For motor fluctuatons**
Dopamine agonists	Pramipexole Pramipexole (ER)	0.125–4.5 mg 0.375–4.5 mg	Drowsiness, nausea, vomiting,dizziness, leg swelling		Efficacious; clinicallyuseful	Efficacious; clinicallyuseful	Efficacios ; clinically useful
	Ropinirole Ropinirole (ER)	0.25–12 mg 2–24 mg	Drowsiness, nausea, vomiting, abdominal discomfort, dizziness, legswelling		Likely efficacious; possibly useful clinically	Efficacious; clinicallyuseful	Efficacio us; clinically useful
	Rotigotine (patch)	2–8 mg	Patch application site reactions (swelling, redness or itching), leg swelling, dizziness, drowsiness		Efficacious; clinicallyuseful	Efficacious; clinicallyuseful	Efficacio us; clinically useful
	Apomorphine sublingual 10-100 mg/day	3–30 mg (intermittent) 16–72 mg (continuous)	Drowsiness, dizziness, lightheadedness, nausea, sweating				Efficacious; clinically useful
Carbidopa/levodopa	Standard levodopa formulation	300–1000 mg	Dyskinesias, nausea, chest pain, cardiac irregularities, vomiting, drymouth	Insufficient evidence on efficacy; investigational implications	Efficacious; clinicallyuseful		Efficacio us; clinically useful
	Levodopa (ER)	855–2205 mg	Dyskinesias, dizziness, nausea, vomiting, insomnia, headache, sweating, salivation		Efficacious; clinicallyuseful		Efficacio us; clinically useful
	Levodopa gel	600–1800 mg	Dizziness, nausea, vomiting,				Efficacio

	intestinal infusion		troublesleeping, headache,				us; clinically useful
	Levodopa inhalationpowder	42–84 mg	Nausea, headache, cough, dyskinesia		Efficacious clinicaluseful		Efficacious rescue therapy
COMT inhibitors	Entacapone	600–1600 mg	Dyskinesias, dizziness, nausea, vomiting, diarrhoea, hallucination,drowsiness, dry mouth, abdominalpain, urine becomes orange			Non-efficacious; clinically not useful	Efficacious; clinically useful
	Tolcapone	300–600 mg	Dyskinesia, dizziness, nausea, vomiting, diarrhoea, hallucination,drowsiness, urine becomes orange			Non-efficacious; clinically not useful	Efficacious; possibly useful clinically
	Opicapone	50 mg	Dyskinesia				Efficacious; possibly useful clinically
MAO-B inhibitors	Rasagiline	1 mg	Dizziness, drowsiness, heartburn, nausea	Insufficient evidence on efficacy; investigational implications	Efficacious; clinicallyuseful	Efficacious; possibly clinically useful	Efficacious; clinically useful
	Selegiline	5 mg	Dizziness, drowsiness, nausea, weightloss	Insufficient evidence on efficacy; investigational implications	Efficacious; clinicallyuseful	Efficacious; possibly clinically useful	Efficacious; clinically useful
	Safinamide	50–100 mg	Dizziness, drowsiness			Possibly clinically useful	Efficacious; clinically useful
Others	Amantadine Amantadine ER	100–300 mg 68.5–274 mg	Dizziness, hallucination, nausea,confusion, myoclonus, livedo reticularis, leg swelling,		Likely efficacious; possibly useful clinically	Likely efficacious; possibly useful clinically	Efficacious for dyskinesias
	Osmolex ER	129–258 mg	Dry mouth, constipation, urinary retention, hair loss, potential exacerbations of heart failure				
	Istradefylline	20–40 mg	Involuntary muscle movements, dizziness, constipation, nausea, hallucination, insomnia		Nonefficacious; clinically not useful		Likely efficacious; possibly useful clinically
	Trihexyphenidyl	2–8 mg	Cognitive impairment, dry mouth,		Efficacious for		

		blurring of vision, urinary retention	resttremor
	Botulinum toxintype A (ona- or incobotulinumtoxin A)	10–100 U Injected in FCR, FCU	Transient hand weakness	Efficacious for rest andaction tremor

(Jankovic and Tan, 2020)

(Grantor, 2021)

Legend: *COMT, catechol-O-methyl- transferase; ER, extended release; FCR, flexor carpi radialis; FCU, flexor carpi ulnaris; MAO-B, monoamine-oxidase type B; PD, Parkinson's disease; s.c. - subcutaneous*

1.4. Lipid droplets

1.4.1. Lipid droplets biogenesis

Lipid droplets are ubiquitous cell organelles whose primary task is to store neutral lipids (Figure 5), while lipid, sterol and phospholipid biosynthesis is a secondary role. Current point of view is that lipid droplets have an additional important role in inflammation, being an inducer of mediator signals or a consequence (or possibly both). That being said, their structure plays a crucial part – core is made of neutral lipids (triacylglycerols and sterol esters), opposed to majority of other organelles with aqueos core, sourended by phospholipid monolayer. With glycerophospholipid acyl chains available, triacylglycerophospholipids start to acculumate, making the hydrophobic core – thus starting lipid droplet biogensis (Figure 4) between the two leaflets of endoplasmic reticulum (ER). Obviously, these processes need to be in presence of enzymes, acyl-CoA:cholesterol O-acyltransferases for sterol esters and diacylglycerol acyltransferases for triacylglicerols to be more precise (Olzmann and Carvalho, 2019).

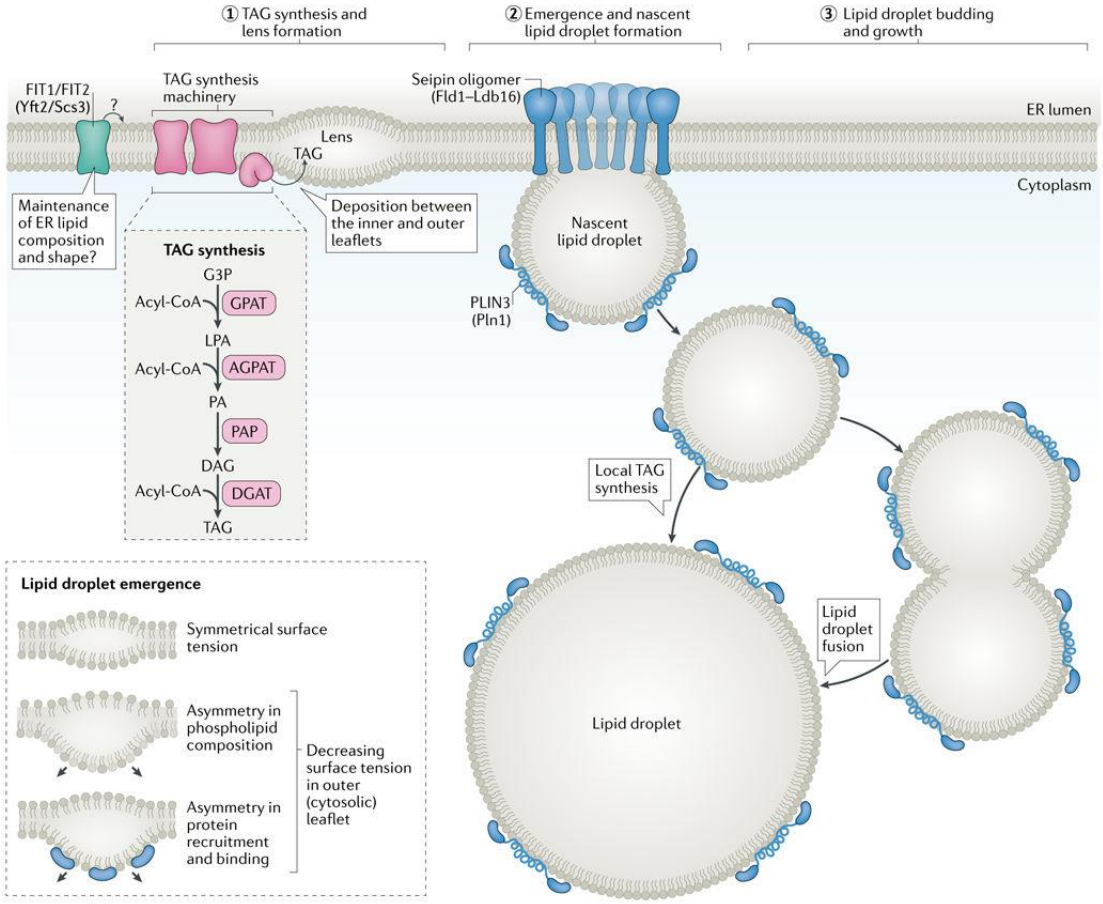


Figure 4. *Steps in lipid droplet biogenesis.*

Lipid droplets emerge from the endoplasmic reticulum (ER). The correct shape and composition of the ER membranes, which are likely affected by the fat storage-inducing transmembrane 2 (FIT2) protein and other ER-resident proteins, are important determinants of organized lipid droplet biogenesis. Step 1: triacylglycerol (TAG) synthesis (see inset) and cholesterol ester synthesis enzymes deposit neutral lipids in between the leaflets of the ER bilayer. Beyond a certain concentration, the neutral lipids demix and coalesce into a lens. Step 2: seipin and other lipid droplet biogenesis factors are recruited to the lens structure and facilitate the growth of the nascent lipid droplet. The emergence of the lipid droplet into the cytosol is affected by differences in surface tension of the luminal and cytosolic leaflets of the ER bilayer, likely determined by asymmetrical protein binding and phospholipid composition (shown in inset). Step 3: in some mammalian cells, lipid droplets bud from the ER and grow through fusion or local lipid synthesis. AGPAT, acylglycerolphosphate acyltransferase; DAG, diacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; G3P, glycerol-3-phosphate; GPAT, glycerol-3 phosphate acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PLIN, perilipin. Yeast orthologues of mammalian proteins are given in parentheses. (Olzmann and Carvalho, 2019)

Second occurrence between those leaflets is forming oil lenses throgh lipid demixing. In that event formation is very fragile, so apolipoprotein B-100 has a task to stabilize that intermediate. Next, ER cytolosic leaflet coats these neutral lipid structures which then bud toward the cytosol. As lipid droplets form on the surface of ER, they are slowly reaching their critical concentration and the energy barrier which needs to be crossed for their release. They are physically bound to ER by lipidic bridges until then. What presented a huge factor is membrane curvature among many things, although exact mechanism how it lowers engergy barrier is still unknown. (Santinho et al., 2020)

In line with that, sterol esters and triacylglycerols may take different comfromations to further lower the energy barrier. Additionally. TAG-s may form clusters and resorb rapidly to slow down their own diffusion into cytosol and with that reach the critical concentration needed for lipid droplet nucleation. Despite the possibility to detach, fluorescence imaging suggests that approximately 85% of lipid droplets remain in contact with the ER in mammalian cells. (Olzmann and Carvalho, 2019) Nonetheless, lipid droplets can also reattach to the ER membrane, counting that COPI coatomer complex is present. Small GTPase ARF1 and its guanine nucleotide exchange factor recruits the complex to lipid droplets and will support detachment of very small lipid droplets. With surface-to-volume ratio severely raised, surface tension is massive and that provokes lipid droplet-surface fusion. Budding is expedited by family of proteins called perlipins. In human body there are 5 types of perilipin (Pln 1-5) with a crucial role to regulate LD stability by inhibting lipolysis. Several other proteins are important at LD biogensis regulation, such as Seipin, Pex30, FIT2. Seipin has a huge role in budding proccess and in incorporation of proteins and lipids into lipid droplets. Without it, budding is restricted and morphology of LDs is tempered. Deletion of BSCL2 (encoding seipin) in human cells disrupted the morphology of ER-lipid droplet contacts and some lipid droplets completely detached from the ER. However, another study found that seipin depletion did not disrupt ER-lipid droplet tethering, suggesting that additional proteins contribute to the ER-lipid droplet contacts. Additional tethering components at the ER-lipid droplet contacts include DGAT2, which pairs with fatty acid transport protein 1 (FATP1), RAB18 together with the NAG-RINT1-ZW10 (NRZ) complex and its associated sNAREs (Syntaxin 18, USE1 and BNIP1)81 and inheritance of cortical ER protein 2 (Ice2). Although deletion of RAB18 affected lipid droplet growth in cultured adipocytes, it had little effect in several other cell lines indicating

that tethering mechanisms for lipid droplets may be context-specific. (Nettebrock and Bohnert, 2020)

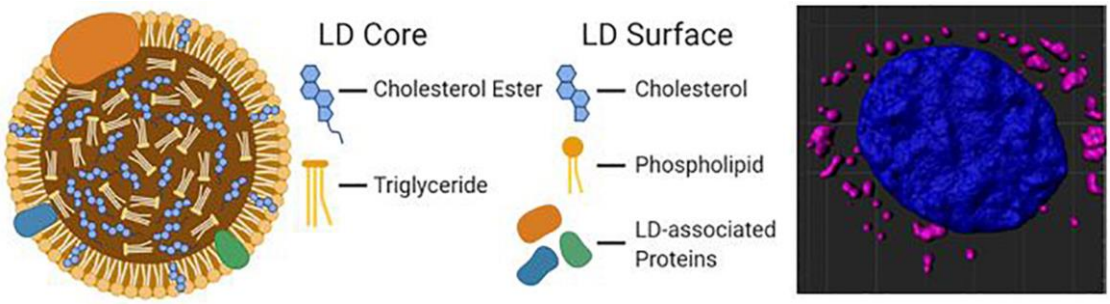


Figure 5. *A diagram of the molecular structure of a standard lipid droplet (LD) along with a software reconstruction of a microscopic image of astrocytes in vitro containing lipid droplets (highlighted in pink; LipidSpot) surrounding a nucleus (in blue; DAPI*

(Farmer, 2020)

1.4.2. Lipid droplet function

Naturally, main lipid droplet involvement is in lipid metabolism, but other than that, they participate in protein debasement and membrane transport. Proteins entangled with lipid droplets are absent of specific targeting signal in their sequence. By origin source lipid droplet proteins are placed in 2 classes: 1. Class 1 – proteins which distribute between ER and lipid droplets; 2. Class 2 – proteins which are mobilized directly from cytosol. Some of the proteins belonging to class 1 are involved in biogenesis, such as long-chain-fatty-acid-CoA ligase 3 ACSL3), while some participate in ubiquitin-depended proteolysis, for instance ancient ubiquitus protein (AUP1). These class 1 proteins are linked with membranes trough hairpins, being inside the bilayer halfway, where both terminal amino and carboxy groups are facing the cytosol. There is a possibility that these hairpin conformations pose as instability conformations and are as such recognised by Endoplasmic reticulum inside ER-associated protein degradation. On the other hand, class 2 proteins bind to lipid droplets through amphipathic α helices. Latest studies shown that phospholipid monolayers are quite susceptible to packing defects where their surface properties are conveniently customized by integrating neutral lipids from the core to the surface. (Olzmann and Carvalho, 2019) Lipid droplets are fundamental in maintaining ER homeostasis by buffering fatty acids and inhibiting unfolded protein response (UPR). UPR is started in ER stress, for instance caused by neurodegenerative disease, where it leads to enhanced microglial activation, synaptic

dysregulation, direct cell toxicity by accumulating unfolded proteins and lipids, and other pathogenic processes. UPR can be upregulated if LD biogenesis is disturbed or if lipid droplet storage capacity is overcrowded. Furthermore, lipid droplets have an important role in containing pathological autophagy. In lack of nutrients autophagy is induced which leads to rise in numbers of free amino acids and lipids, all under mTOR complex 1 management. Some of these lipids are esterified again into triacylglycerol in ER (transport to ER is still unknown) and packed into lipid droplets. Although, energy-wise, synthesis of new lipid droplets may not be the most efficient process, it was found that lipid droplets prevent lipotoxic damage to mitochondrial membrane posing as a buffer. Impaired lipid droplet biosynthesis can lead to advanced mitochondrial membrane potential dysregulation, weakened mitochondrial respiration and intensified cell death. Fatty acids are required in mitochondrial ß-oxidation and their recruitment to mitochondria is regulated by acylcarnitine which fail to be synthetized in inadequacy of lipid droplets. It is interesting to observe that autophagy-dependent lipid droplets are DGAT1 specific. The question arises; what causes lipid droplets to form in brain? Well, natural cause is aging. With progressed age, lipid droplet count surely rises proportionally. In a recent study, lipopolysaccharide (LPS) was used to cause inflammation in hippocampus and lipid droplet count in microglia was compared to an aging microglia lipid droplet count where the results showed quite similar situation. It is not only quantitative LD regulation that age is adjusting, but also the placement, studies has shown that lipid droplets have been shifting from perivascular cells to the parenchyma with age. Lipid droplets seems to be the cause and the consequence of inflammation, producing cytokines as inflammation mediators, and later in the process lipid droplets are being upregulated inside that same inflammation. Reactive oxidative species, as well as other oxidative stress mediators, also induce lipid droplet biogenesis. While specific mechanism behind reason of lipid droplet appearance in neurodegenerative diseases is still unknown, they seem to be present, and they seem to have a much bigger role than previously understood. Huntington’s disease, Amyotrophic lateral sclerosis, Hereditary spastic paraplegia, Alzheimer’s disease, Parkinson’s disease etc. all of them share the lipid droplet abundance (Mohimani, 2017).

2. Research objectives

Both Parkinson's disease and stroke make a highly difficult treatment targets, and in lack of treatments with acceptable survivability, new approaches are emerging. Lipid droplets are well-defined organelles, yet in brain they show some specific features. They seem to be one of the crucial parts during neuroinflammation paired with microglia, as they have been identified in an aging brain. (Mohimani et al., 2017)
This thesis is the beginning of a larger study in progress aimed to define exact role of lipid droplets in neurodegenerative diseases and determine if they would be a suitable drug target. Parkinson's disease and ischemic stroke are the targets diseases in this thesis.
There are few aims of this thesis. Firstly, lipid droplets need to be properly stained with green fluorescent protein (GFP) dye to identify them. Focus is on visualisation and potential quantification of lipid droplets. Next, lipid droplets are visualized and quantified in cell cultures.

To optimize GFP staining, several concentrations of the dye are tested and then compared by quality of visualization and amount of autofluorescence. To further visualize lipid droplet, microglia and neurons are being stained, and therefore droplets can be colocalized with them. Quantification is based on comparison of percentage of area covered with lipid droplets between stroke influenced side of the brain and non-influenced side using symmetrical sites regarding fissure. Comparison is made between samples with different treatment. Naloxone was used as it proved to reduce stroke caused inflammation and degeneration of neurons (Anttila et al., 2018).

To investigate treatments on cell culture, lipid droplets are being counted and compared in differently treated samples. The hypothesis was that lipid droplets will emerge in early stages of states of neuroinflammation.

3. Materials and Methods

3.1. Parkinson's model

Mice of randomized age were injected with 6-hidroxydopamine (6-OHDA) in striatum and brains were sectioned and postfixed in 4% paraformaldehyde for 2 days, dehydrated in series of ethanol and xylene and embedded in paraffin. Brains were cut into 30-µM-sections using Leica HM355S microtome. Sections were previously prepared inside same laboratory group (Anttila et al., 2018).

6-OHDA was injected 3, 7, 14, 21 and 28 days after lesion, specifically dorsomedial part of striatum. Briefly, animals were anesthetized with chloral hydrate 0.4 g/kg intraperitoneally (i.p.) as the body temperature was kept at 37°C. After recovery from anesthesia, rats were returned to their home cage. Drugs were administered intranasally under isoflurane anesthesia (Anesthesia Auto Flow System; E-Z Anesthesia) starting from post lesion day 1 up to day 28. As our interest was to observe lipid droplets in inflammation, 3 days post lesion sections of striatum were used. Rains were dehydrated in 30% sucrose at 4°C and sectioned coronally into 30-µm-thick slices using a Leica CM3050 Cryostat. Sections were taken from 2.0 to –1.0 mm (striatum) and –2.4 to –4.1 mm (thalamus) relative to bregma, then stored in 1xPBS for short-term storage or cryopreservant for long-term storage (20% glycerol, 2% DMSO in 1x PBS). 129 inbred mice model was used. Experimental procedures were approved by the NIDA Animal Care and Use Committee or by the National Animal Experiment Board of Finland and followed the guidelines of the "Guide for the Care and Use of Laboratory Animals" and local laws and regulations (Airavaara, 2020).

3.2. Stroke model

To model focal cortical ischemic stroke in rats, the three-vessel occlusion was used. In this model, the stroke damage is restricted to cortex, and the relative size of the stroke is close to that of an average human stroke. (Delavaran et al., 2013) Briefly, rats were anesthetized with chloral hydrate 0.4 g/kg intraperitoneally (i.p.). The bilateral CCAs

were identified and isolated through a ventral midline cervical incision. The rats were placed in a stereotaxic apparatus, and a craniotomy was performed to expose the right MCA. The MCA was ligated with a 10-0 suture, and bilateral CCAs were ligated with nontraumatic arterial clamps. After 60 or 90 min of ischemia, the suture around the MCA and arterial clips on CCAs were removed to begin reperfusion. After recovery from anesthesia, rats were returned to their home cage. Body temperature during and after surgery was maintained at 37°C. Drugs were administered intranasally under isoflurane anesthesia (Anesthesia Auto Flow System; E-Z Anesthesia) starting from post-stroke day 1 and were continued at 12-h intervals for 7 d, a total of 14 times. Briefly, animals were placed in the induction chamber for 1–1.5 min, and 5% isoflurane was delivered at 1000 cc/ min. Animals were transferred to the nose cone where they received 1.5% isoflurane delivered at 500 cc/min for 30 s before intranasal naloxone delivery. To ensure maximal delivery, animals remained in supine position during naloxone administration. When the animals regained consciousness, they were returned to the home cage. Naloxone solution was prepared fresh daily in sterile ultrapure water and stored at room temperature between administrations. Using a Rainin LTS L-20 pipette and sterile pipette tips, 10 μL drug or vehicle was administered into each nostril as described (Luo et al., 2013). Rains were dehydrated in 30% sucrose at 4°C and sectioned coronally into 40-μm-thick slices using a Leica CM3050 Cryostat. Sections were taken from 2.1 to –1.0 mm (striatum) and –2.4 to –4.0 mm (thalamus) relative to bregma, then stored in 1xPBS for short-term storage or cryopreservant for long-term storage (20% glycerol, 2% DMSO in 1x PBS). Adult male Sprague-Dawley rats (200–250 g; Charles River) were maintained under a 12-h light-dark cycle. Food and water were freely available in the home cage. Experimental procedures were approved by the NIDA Animal Care and Use Committee or by the National Animal Experiment Board of Finland (protocol approval number ESAVI/5459/04.10.03/2011) and followed the guidelines of the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication, 1996) and local laws and regulations (Anttila et al., 2018).

3.3. Immunohistochemistry

a) Tissue samples

Immunostaining of mice striatum free floating sections was performed with fluorescent neutral lipid dye 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503) and nuclei were dyed with
4',6-diamidino-2-phenylindole (DAPI). BODIPY (Figure 6.) is a green emitting fluorescent dye with ability to stain neutral lipids and trace oil and nonpolar lipids.

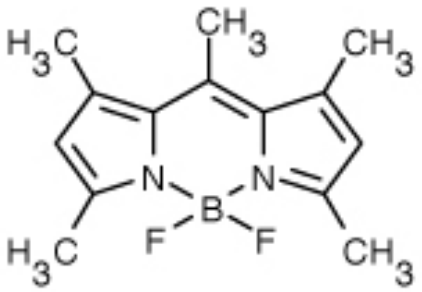


Figure 6. *BODIPY 493/503 structure*

Sections were first washed in phosphate buffered saline (PBS) and then embedded in 4% paraformaldehyde (PFA) for 5 minutes, washed with PBS, then blocked with 0,3% Tritonx-100) three times for 20 minutes. After that stained with BODIPY for 30 minutes followed by 3 PBS washes and DAPI staining for 5 minutes. To optimize BODIPY concentration, 1:500, 1:1000, 1:2000 and 1:5000 concentrations were tested depending on fluorescence signal visualization possibility and background fluorescence which needs to be avoided. Then sections were mounted on slides and left to dry at least 3 hours on room temperature. After that, cover slips were mounted on top using Fluoromount-G® and samples were stored on +4°C in conditions with minimal light and microscoped using Nikon fluorescence microscope. Meanwhile, information that Triton X-100, non-ionic surfactant, which was used for permeabilization, could cause destabilization of lipid droplets so it was later discarded out of the immunohistochemistry process. Next, rat thalamus was treated through the same process (without permeabilization). To optimize visualization further, other organelles were stained for immunofluorescence imaging. To identify neurons NeuN biomarker was used, immunostaining it with mouse anti-NeuN as a primary antibody, and as a secondary antibody donkey anti-mouse Immunoglobulin G (IgG) was used. Neurons were marked so only droplets inside cells could be precisely pinpointed. Furthermore, to locate inflammation site along which microglia migrate there, IBA1 marker was used,

immunoassayed with rabbit anti-IBA1 primary antibody. As a secondary antibody, goat anti-rabbit igG was used. Samples were assayed in primary antibody solution overnight and for an hour in secondary antibody solution in +4°C. Drying process after mounting samples onto slides was also discarded to avoid any air pollution between mounting and coverslipping. DAPI and BODIPY staining remained same. Standard light fluorescence microscope was used in BODIPY optimization process and estimation of lipid droplet existence location. Further, Confocal microscope was used properly visualize and confirm lipid droplet existence in samples and slide scanner was used to semi-quantify lipid droplets. For stroke tissue samples, two sides of brain were analysed from slide scanner images. One side damaged by stroke, and the other damage-free were compared by area percentage which lipid droplets covered in randomly chosen location on the damaged site and then symmetrical location regarding brain fissure. Results were analysed using GraphPrism Wilcoxon matched pairs signed rank test.

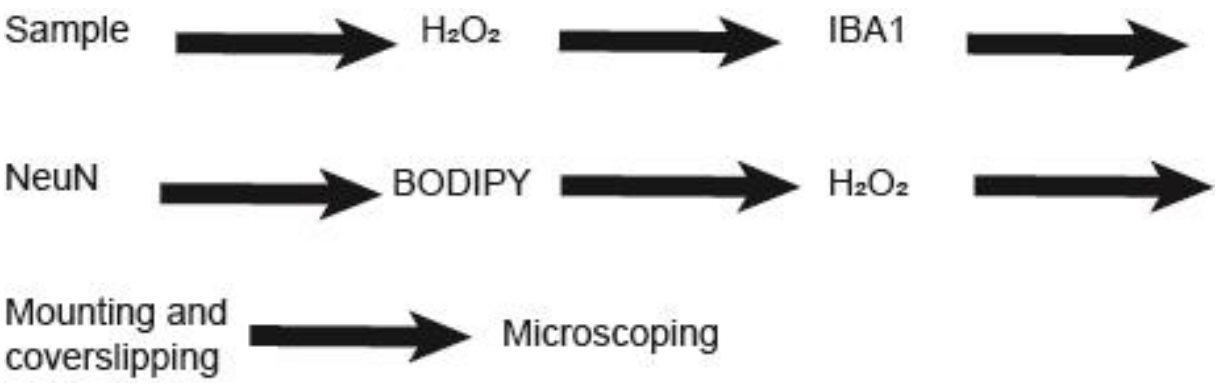


Figure 7. *Immunostaining process*

b) Cell culture

To check in lipid droplets are indeed overly synthetized in microglia inflammatory states, BV2 cells (which are a microglia model cell line) were embedded in thapsigargin in concentrations of 0.1, 0.5, 1 mg/mL (plus control without any thapsigargin) for four hours. Cells were then washed in PBS for five minutes, fixed in 4% PFA for five minutes, washed two times in PBS for 5 minutes, permed with 2% bovine serum albumin and 0,3% TritonX-100. After that, cells are again washed three times in PBS for five minutes, followed by incubation with BODIPY dye (1:1000) for fifteen minutes, three PBS washes of five minutes and, finally, nuclei stained with DAPI (1:1000) for ten minutes in dark. Whole experiment is done at room temperature. Cells were then

mounted onto slides, coverslipped and microscoped using slide scanner and confocal microscope. Number of lipid droplets inside cells was determined using ImageJ counter and averaged (N=4). Additionally, influence of permeabilization step was tested through comparation of lipid droplet number in cells treated with and without 4% BSA and TritonX-100 (N=4).

Figure 8. *Experiment process*

Key Materials

Reagent or resource	Source	Identifier
BODIPY 493/503	Thermo Fisher Scientific	Cat# D3922
Thapsigargin	Thermo Fisher Scientific	Cat# T7459
DAPI	Thermo Fisher Scientific	Cat# D1306
TritonX-100	Sigma Aldrich	Cat# T9284
Mouse anti-NeuN	Abcam	Cat# ab104224
Rabbit anti-Iba1	Abcam	Cat# ab178846
Sprague-Dawley rat	Generated in house	(Anttila et al., 2018)
BV2 cell line	Generated in house	
Prism	GraphPad	https://www.graphpad.com
ImageJ	National Institute of Health	https://www.nih.gov
Fiji	Fiji	https://fiji.sc
Adobe Illustrator CC 2019	Adobe Inc.	https://www.adobe.com/products/illustrator.html
CaseViewer	Histech Ltd.	https://www.3dhistech.com/solutions/caseviewer/
Huygens Professional	Scientific Volume Imaging	https://svi.nl/Huygens-Professional
Nikon Fluorescence miscroscope	HiLIFE (In house)	https://hilife.science-it.ch/Landing/Resource/6504
PerkinElmer Opera Phenix microscope	FIMM (HiLIFE)	https://www2.helsinki.fi/en/infrastructures/bioimaging/fimm-hca/microscopes-and-software
GBU slide scanner	Genome biology unit (HiLIFE)	https://www2.helsinki.fi/en/researchgroups/genome-biology-unit/scanning
Zeiss Axio	Biomedicum Imaging Unit	https://www2.helsinki.fi/en/infrastructures/bioimaging/biu/instruments/widefield-microscopes

Imager 2 with Apo-Tome		
Leica TCS SP8 X	Biomedicum Imaging Unit	https://openiris.io/Landing/Resource/3875

4. Results and discussion

First visualizations of Parkinson's model samples were less than ideal, showing that staining optimizations were needed. Several problems came into picture; 1. BODIPY concentrations; 2. Samples thickness; 3. Bad visibility due to microscope attributes; 4. Drying process after mounting; 5. Surfactant Permeabilization. Firstly, BODIPY dye concentrations needed to be sorted. 1:500, 1:1000, 1:2000 and 1:5000 concentrations (from 1 mg/mL) were tested. Huge obstacle was background autofluorescence where brightness increased with BODIPY concentrations. Therefore, main task was to reduce autofluorescence, while visibility of the droplets should remain intact (if possible). After number of images, conclusion was that 1:1000 concentration (Figure 9) shows the right balance, although thickness of those 6-OHDA model samples was probably something which caused them to be inapplicable in visualizing lipid droplets. That is why those samples were not used in the remaining experiments, but were sufficient for concentration optimization.

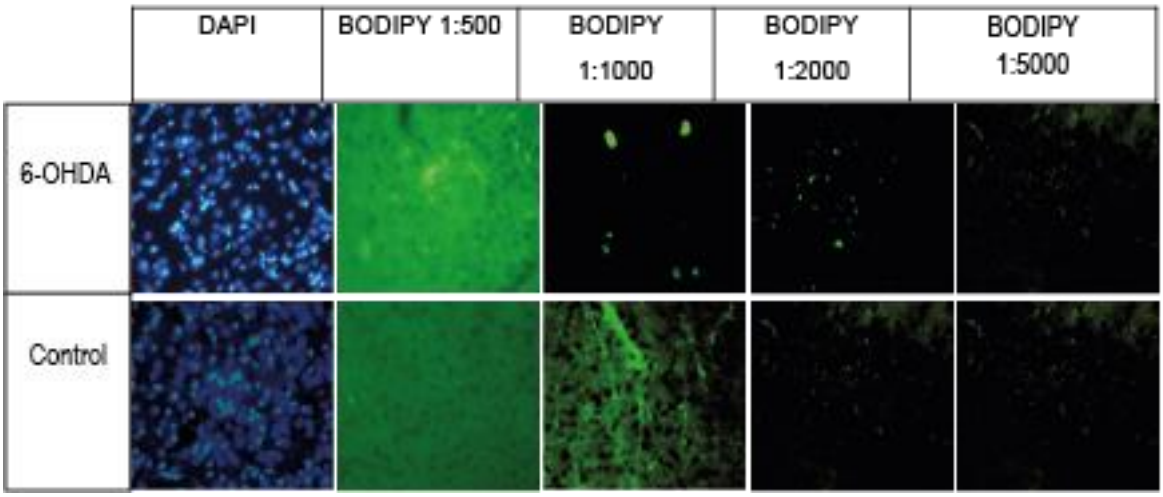


Figure 9. *Optimization of BODIPY dye; blue are DAPI stained nuclei, green represents BODIPY. In Control samples striatum is not treated with 6-OHDA. It is visible how autofluorescence is high when concentration is high. There is no clear difference between lipid droplets, background and miscellaneous impurities. Images are taken with 40x magnification.*

Meanwhile, rat brain samples fourteen days after inducing stroke (they were frozen at this timepoint - Anttila et al., 2018) showed better progress – using anti-Iba1 and anti-NeuN visualization advanced onto another level. Moreover, samples provided clearer images, although not without abundance of background signal. Using Confocal microscope and 3D reconstruction, there was definite evidence of colocalization (Figure 10.), but as this was qualitative research, there is no significant statistical quantification provided inside this thesis.

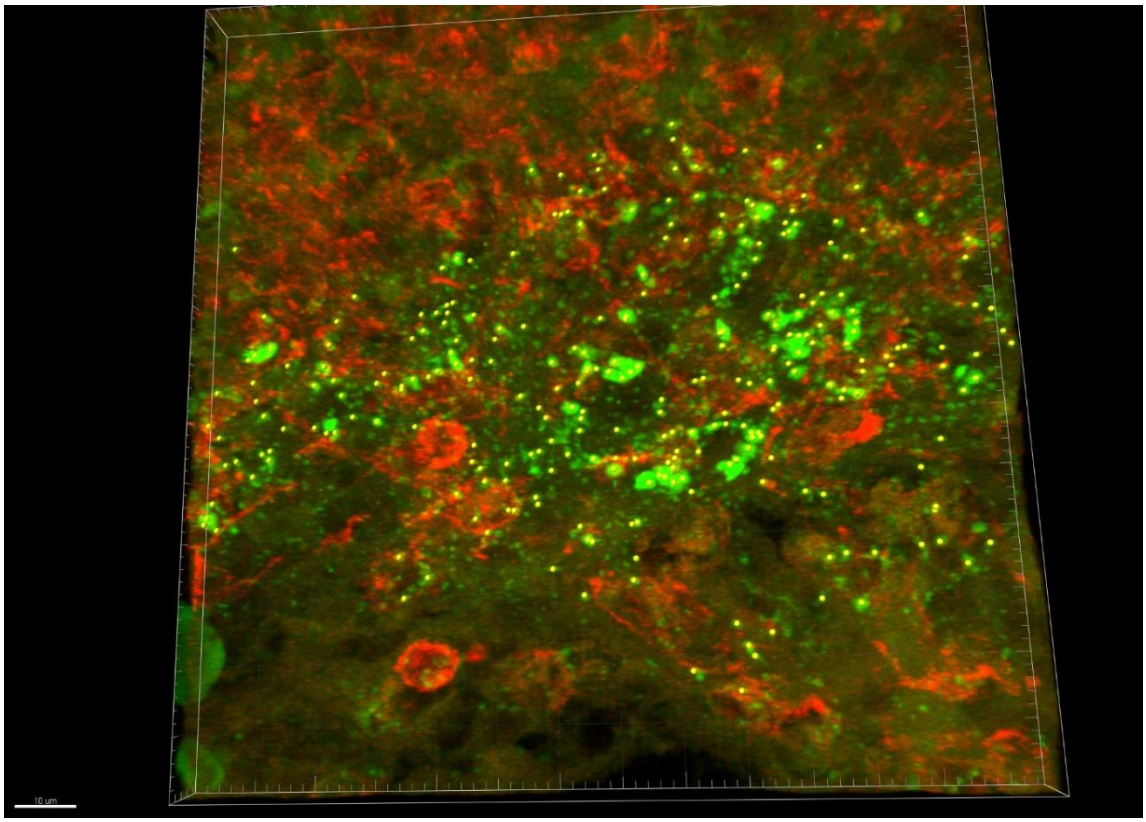


Figure 10. *Non-treated stroke sample marked with anti-Iba1 (red) for microglia and BODIPY (green) for lipid droplets. It's a 3D reconstruct made in Huygens professional and it is visible that colocalization of microglia and lipid droplets exist. It was imaged with Zeiss Axio Imager 2 with Apo-Tome on 60x1000 magnification.*

Further, those same samples were imaged using slide scanner, scanning surface on randomized location y-axis wise. Images were taken on symmetrical sites of hemispheres as control. Images were compared through percentage of area that lipid droplets covered. Respectively, it is not an entirely quantitative analysis, but it presents difference between those two sides of the brain, the difference between brain with and without induced stroke, but also between Naloxone treated, non-treated and purified water treated brain. While images show "green-filled" areas and areas without "green droplets", it should be stated that quantitative measurements are still required. Later figures show these differences (Figure 11-14)

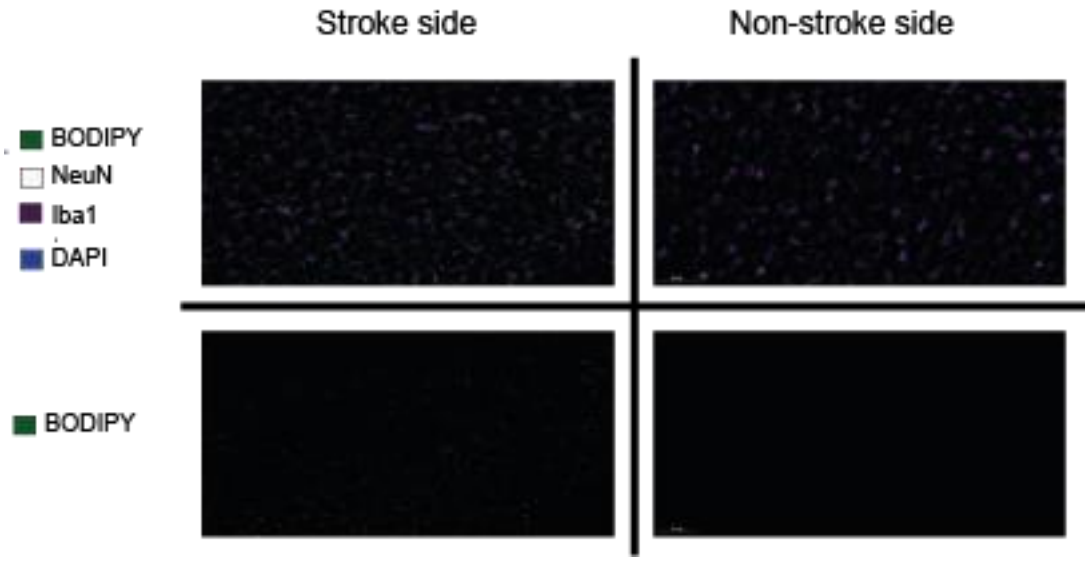


Figure 11. *Brain thalamus without treatment 14 days after inducing stroke. Symmetrical locations on hemispheres are shown on 30.8x1000 magnification. Upper and lower images are the same with all other stains other than BODIPY filtered out.*

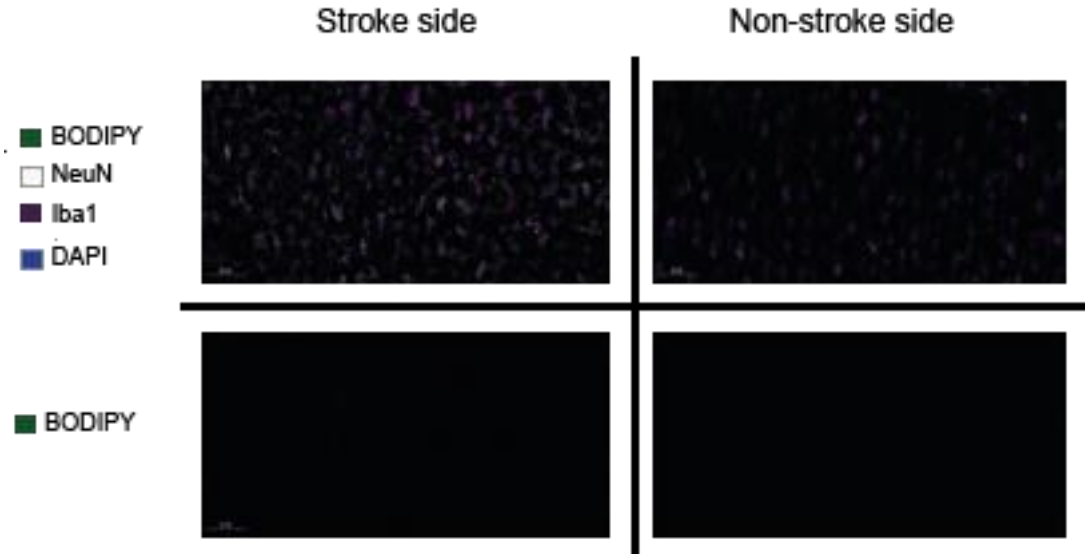


Figure 12. *Brain thalamus 14 days after inducing stroke treated with Naloxone 10 mg/kg. Symmetrical locations on hemispheres are shown on 30.8x1000 magnification. Upper and lower images are the same with all other stains other than BODIPY filtered out.*

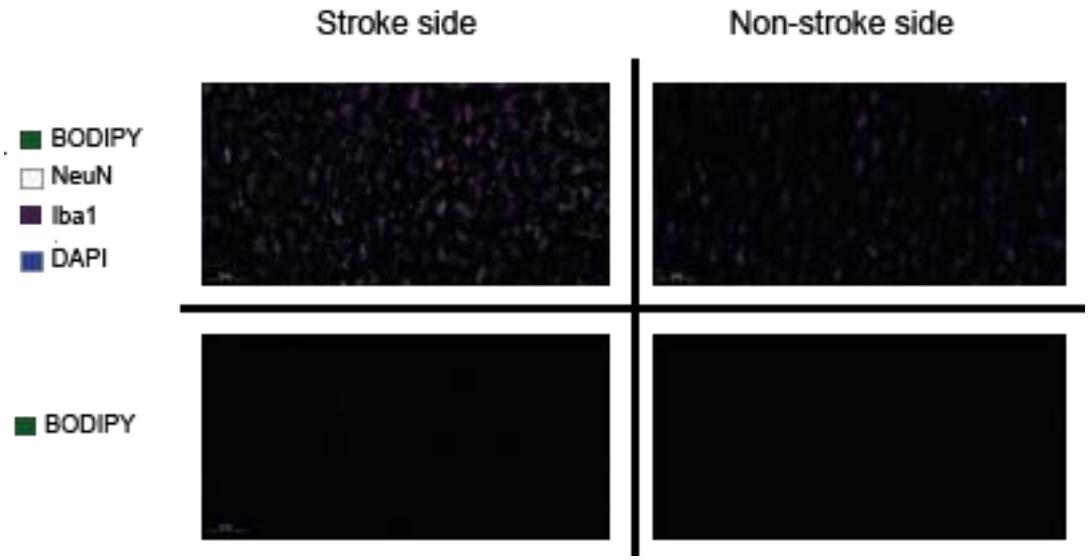


Figure 13*. Brain thalamus 14 days after inducing stroke treated with purified water. Symmetrical locations on hemispheres are shown on 30.8x1000 magnification. Upper and lower images are the same with all other stains other than BODIPY filtered out.*

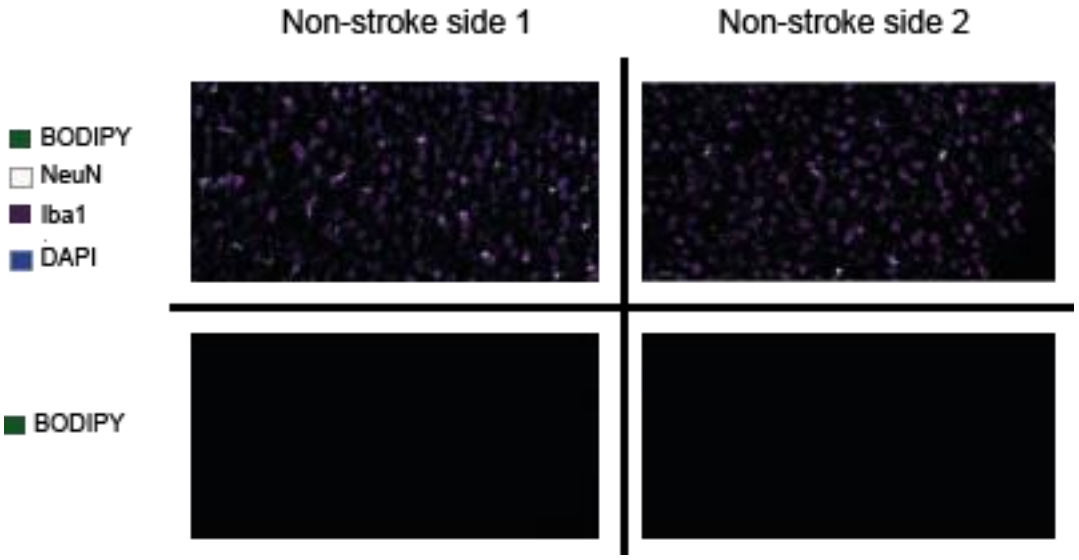


Figure 14*. Naïve brain thalamus without induced stroke. Symmetrical locations on hemispheres are shown on 30.8x1000 magnification. Upper and lower images are the same with all other stains other than BODIPY filtered out.*

Imaging results show a contrast between inflamed stroke brain and brain without induced stroke. Abundance of lipid droplets can not be denied and, while it is clear that lipid droplets have a role here, it is not clearly defined which role is it.

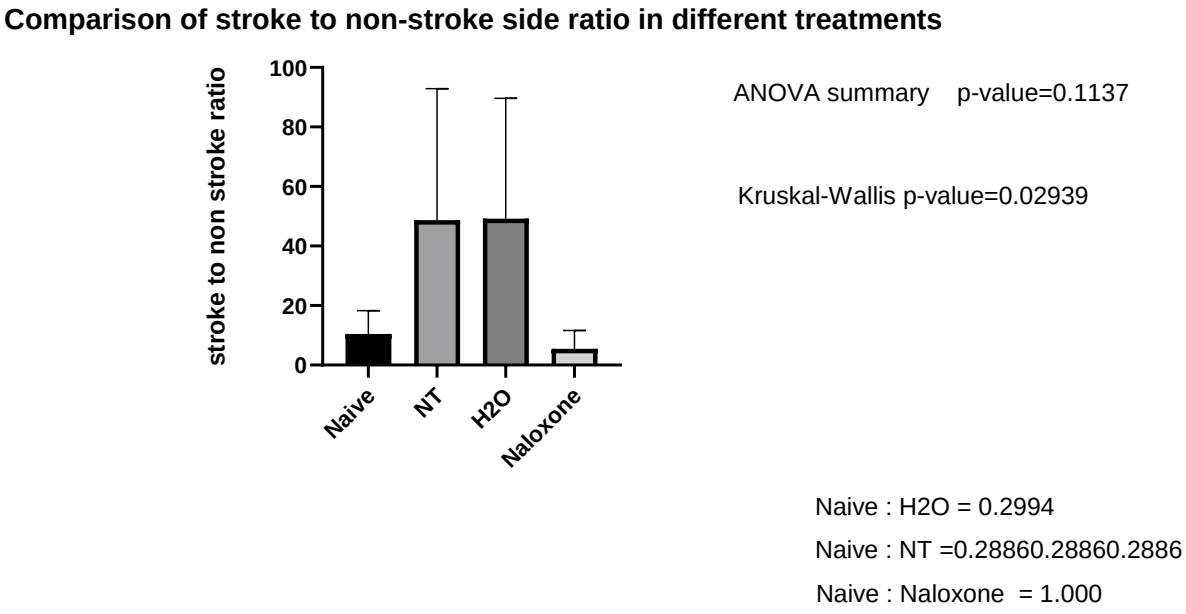


Figure 15. *Symmetrical locations on two brain hemispheres are compared via area percentage that lipid droplets cover. Locations are chosen randomly on a 30.8x1000 magnification. These images are then compared with rest of the treatments using ANOVA and Kruskal-Wallis tests. P<0.05 shows a significant result. Area percentage comparison relative to naïve sample is made in bottom right corner, H2O – stroke samples treated with purified water, NT- non treated stroke samples and Naloxone – naloxone (10 mg/kg) treated stroke samples.*

While ANOVA summary shows a high p value and thus it is a result of low significance, and Kruskal-Wallis shows that the result is significant (Figure 15.). Because of the type of results and number of variables, Kruskal-Wallis is the one more applicable to this experiment. As this method was semi-quantifiable, these p values should not be considered as an absolute result of the experiment. Interesting element to consider is the ratio between different treatments. Non-treated and water treated samples show almost 4-time value compared to naïve sample and naloxone treatment (Figure 15.), which was expected in hypothesis. Afterward, cell culture samples were analysed and compared through number of lipid droplets in randomly chosen cells. Comparison was made between cells treated with 1 μM, 0.5 μM, 0.1 μM Thapsigargin and a control without Thapsigargin (Figure 16.).

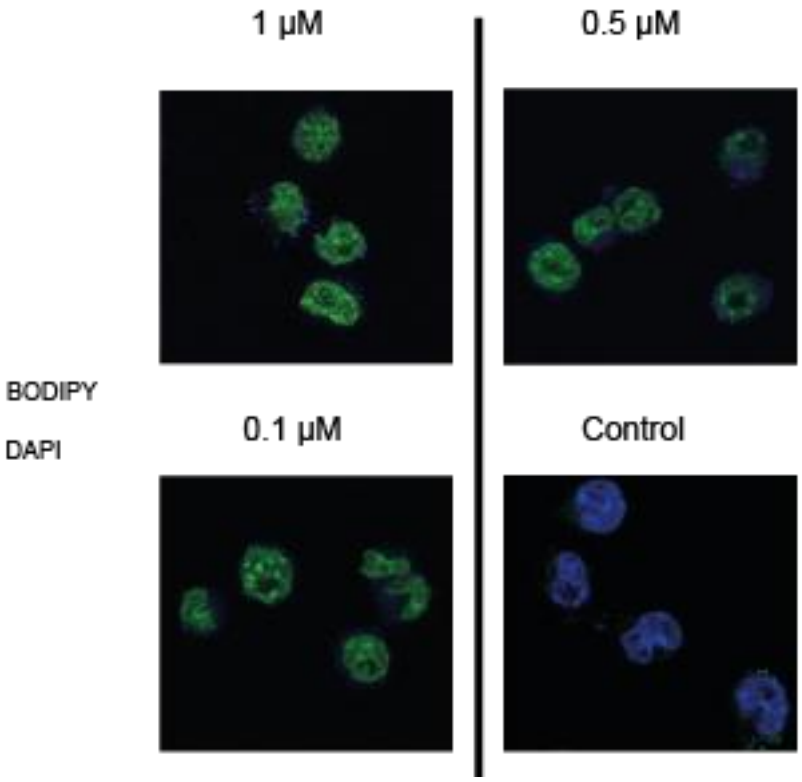


Figure 16. *BV2 cells treated with thapsigargin in different concentrations (1 µM, 0.5 µM, 0.1 µM) compared with control without thapsigargin. Locations are randomly chosen on magnification of 904x904.*

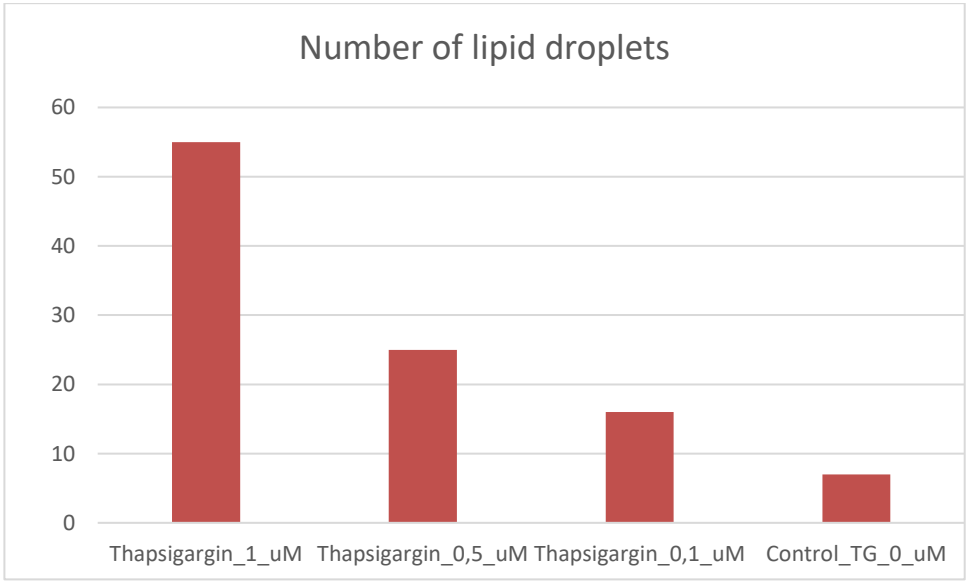


Figure 17. *Number of lipid droplets compared throughout the samples with number of cells in which lipid droplets were counted using ImageJ and averaged of four (N=4).*

Images stained with BODIPY, and counter stained with DAPI show a number of lipid droplets present in BV2 cells treated with Thapsigargin with a considerable decrease in number as

concentration is dropping (Figure 18.). Figure 1 reveals true numbers and confirms previous hypothesis. It is important to say that control samples also contain small number of lipid droplets which confirms lipid droplet biogenesis outside inflammation statements.

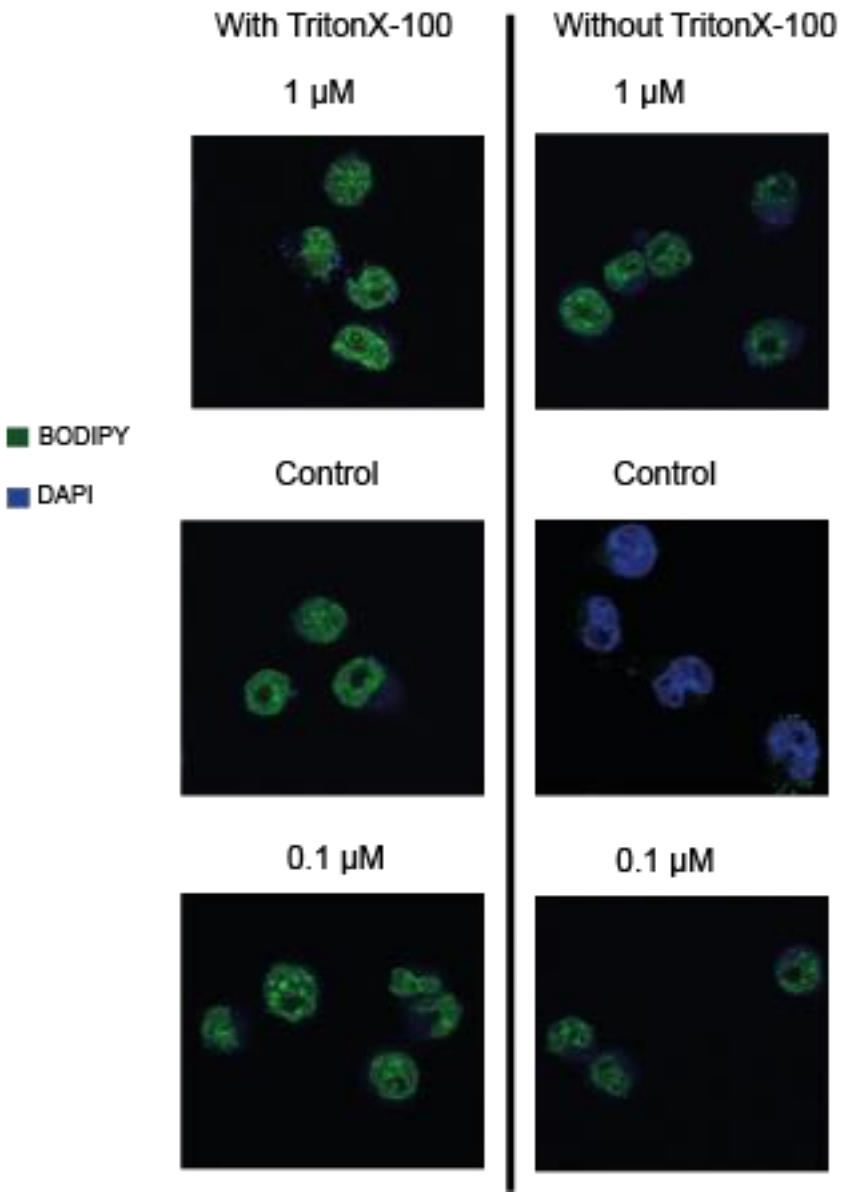


Figure 17. *BV2 cells treated with thapsigargin in different concentrations (1 µM and, 0.1 µM) along with control without thapsigargin compared to cells treated in the same manner with additional 0,3% TritonX-100 permeabilization. Locations are randomly chosen on magnification of 904x904.*

By comparing images of cells permeabilized with TritonX-100 and cell which were not permeabilized, there were no significant difference as opposed to previous thoughts of the surfactant decreasing number of lipid droplets.

5. Conclussion

Although immunohistochemical results on 6-OHDA Parkinson's disease model samples were inconclusive, BODIPY as a dye used for staining lipid droplets has been confirmed as a good choice. In other experiments, increased number of lipid droplets has indeed been affirmed with imaging, thus supporting hypothesis of lipid droplet role in stroke. While exact role is yet to be established, and it is still not clear if lipid droplets are cause or consequence, or maybe even both, in inflammation, their significance can not be denied. As such, they could make a proper target for treating such states of brain. Further experiments should quantitatively analyse lipid droplet at different timepoints in stroke and Parkinson's disease to determine whether they would indeed be a targetable option in these states. Triton-X 100 experiments showed that there was no lipid droplet dissolving following permeabilization, although this should be further checked using different surfactants.

6. References

Airavaara M, Voutilainen MH, Wang Y, Hoffer B. Neurorestoration. *Park Relat Disord*, 2012, 18, 1–7.

Airavaara M, Parkkinen I, Konovalova J, Albert K, Chmielarz P, Domanskyi A. Back and to the Future: From Neurotoxin-Induced to Human Parkinson's Disease Models. *Curr Protoc Neurosci*, 2020, 91, 1–33.

Anttila JE, Albert K, Wires ES, Mätlik K, Loram LC, Watkins LR, Rice KC, Wang Y, Harvey BK, Airavaara M. Post-stroke intranasal (+)-naloxone delivery reduces microglial activation and improves behavioral recovery from ischemic injury. *eNeuro*, 2018, 5.

Campbell BCV, Khatri P. Stroke. *Lancet*, 2020, 396, 129–142.

Chmielarz P, Er Ş, Konovalova J, Bandres L, Hlushchuk I, Albert K, Panhelainen A, Luk K, Airavaara M, Domanskyi A. GDNF/RET Signaling Pathway Activation Eliminates Lewy Body Pathology in Midbrain Dopamine Neurons. *Mov Disord*, 2020, 35, 2279–2289.

Elbaz A, Carcaillon L, Kab S, Moisan F. EPIDEMIOLOGY OF PARKINSON'S DISEASE The Rotterdam Study. *Rev Neurol (Paris)*, 2015, 1580, 1–13.

Goetz CG. The history of Parkinson's disease: Early clinical descriptions and neurological therapies. *Cold Spring Harb Perspect Med*, 2011, 1

Hosein Mohimani, Alexey Gurevich, Alla Mikheenko, Neha Garg, Louis-Felix Nothias, Akihiro Ninomiya, Kentaro Takada, Pieter C. Dorrestein3 and PAP. Lipid droplet accumulating microglia represent a dysfunctional and pro-inflammatory state in the aging brain. *Physiol Behav*, 2017, 176, 139–148.

Jacobson S, Marcus EM. Neuroanatomy for the Neuroscientist, ISBN-13: 978-0-387-70970-3, Deneme Kitabı Chapter için. 2008,

Jankovic J, Tan EK. Parkinson's disease: Etiopathogenesis and treatment. *J Neurol Neurosurg Psychiatry*, 2020, 91, 795–808.

Jarc E, Petan T. A twist of FATe: Lipid droplets and inflammatory lipid mediators. *Biochimie*, 2020, 169, 69–87.

Nettebrock NT, Bohnert M. Born this way – Biogenesis of lipid droplets from specialized ER subdomains. *Biochim Biophys Acta - Mol Cell Biol Lipids*, 2020, 1865, 158448.

Olzmann JA, Carvalho P. Dynamics and functions of lipid droplets. *Nat Rev Mol Cell Biol*, 2019, 20, 137–155.

Raza C, Anjum R, Shakeel N ul A. Parkinson's disease: Mechanisms, translational models and management strategies. *Life Sci*, 2019, 226, 77–90.

Reich SG, Savitt JM. Parkinson's Disease. *Med Clin North Am*, 2019, 103, 337–350.

Santinho A, Salo VT, Chorlay A, Li S, Zhou X, Omrane M, Ikonen E, Thiam AR. Membrane Curvature Catalyzes Lipid Droplet Assembly. *Curr Biol*, 2020, 30, 2481-2494.e6.

7. Summary

Parkinson’s disease presents a great challenge in diagnose and treatment, as does stroke. Both require attention to improve these treatments and survivability. Somewhat new and uncharted territory in brain are lipid droplets. They are storage organelles as the pillar of lipid and energy homeostasis, but their function in brain is not yet absolutely defined and it could be of great influence. Goal of this thesis is to positively identify lipid droplets as active participants in neuroinflammation process regarding ischemic stroke and Parkinson’s disease using 6-OHDA models for Parkinson’s and three-vessel occlusion samples for ischemic stroke. Additionally, lipid droplets as participants in neuroinflammation are being identified in thapsigargin treated BV2 cells. After analysing these samples, positive lipid droplet identification has been made. There is a huge incidence of lipid droplets colocalized with local microglia in these induced inflammations. These preliminary results could further lead to making lipid droplet an additional target in the treating some of the neurodegenerative states.

8. Strukturirani sažetak

Uvod: Parkinsonova bolest, kao i moždani udar, predstavlja ogromni problem vezano za dijagnostociranje i liječenja. Oba stanja zahtijevaju unaprijeđenje liječenja i smanjenje posljedica bolesti i smrtnosti. Jedno od nedovoljno istraženih i novih segmenata ovih neurodegenerativnih bolesti su lipidni organeli, temeljni stupovi lipidne i energetske homeostaze unutar stanice, no njihova cjelovita uloga u mozgu nije još definirana i mogla bi biti od velikog značenja.

Cilj i metode: Cilj ovog rada je bio identificirati lipidne kapljice kao aktivne sudionike upalnog procesa u mozgu tijekom Parkinsonove bolesti i ishemičnog moždanog udara koristeći životinjske modele 6-hidroksidopamina za Parkinsonovu bolest i uzorke s trožilnom okluzijom za ishemični moždani udar. Također, lipidne kapljice kao aktivni sudionici upalnih procesa u mozgu identificirane su koristeći BV2 stanične kulture tretirane s tapsigarginom.

Rezultati: Poslije analize uzoraka, lipidne kapljice su sa sigurnošću identificirane. Postoji ogroman broj lipidnih kapljica koje su kolokalizirane sa lokalnom mikroglijom kod upalnih procesa u moždanom tkivu.

Zaključak: Ovi pozitivni preliminarni rezultati možda mogu dovesti do toga da lipidne kapljice postanu jedna od meta u liječenju nekih neurodegenerativnih stanja mozga.

9. Basic documentation card

Temeljna dokumentacijska kartica

Basic documentation card

University of Zagreb
Faculty of Pharmacy and Biochemistry
Study: Master of Pharmacy
Department of Pharmacology
Domagojeva 2, 10000 Zagreb, Croatia

Diploma thesis

Lipid droplets in models of Parkinson's disease and stroke

Leo Sabol

SUMMARY

Parkinson's disease presents a great challenge in diagnose and treatment, as does stroke. Both require attention to improve these treatments and survivability. Somewhat new and uncharted territory in brain are lipid droplets. They are storage organelles as the pillar of lipid and energy homeostasis, but their function in brain is not yet absolutely defined and it could be of great influence. Goal of this thesis is to positively identify lipid droplets as active participants in neuroinflammation process regarding ischemic stroke and Parkinson's disease using 6-OHDA models for Parkinson's and three-vessel occlusion samples for ischemic stroke. Additionally, lipid droplets as participants in neuroinflammation are being identified in thapsigargin treated BV2 cells.
The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 36 pages, 17 figures, 1 table and 16 references. Original is in English language.
Keywords: Stroke, Parkinson's disease, Microglia, rats, mice, inflammation, lipid droplets, cell culture
Mentor: Lidija Bach-Rojecky, Ph.D. Full Professor, University of Zagreb Faculty of Pharmacy and Biochemistry
Co-mentor: Mikko Airavaara, Ph.D. Full Professor, University of Helsinki Faculty of Pharmacy
Reviewers: Lidija Bach-Rojecky, Ph.D. Full Professor, University of Zagreb Faculty of Pharmacy
Sandra Šupraha Goreta. Ph.D. Docent, University of Zagreb Faculty of Pharmacy
Ana-Marija Domijan, Ph. D. Associate Professor, University of Zagreb Faculty of Pharmacy
The thesis was accepted: September, 2021

Temeljna dokumentacijska kartica

University of Zagreb
Faculty of Pharmacy and Biochemistry
Study: Master of Pharmacy
Department of Pharmacology
Domagojeva 2, 10000 Zagreb, Croatia

Diplomski rad

Lipidne kapljice u modelima Parkinsonove bolesti i moždanog udara

Leo Sabol

Sažetak

Parkinsonova bolest, kao i moždani udar, predstavlja ogromni problem kod dijagnostociranja i liječenja. Oba stanja zahtijevaju trenutnu pažnju za unaprijeđenje liječenja i smanjenje smrtnosti. Jedno od nedovoljno istraženih i novih dijelova tih bolesti su lipidne kapljice. One su, kao lipidni organeli, temeljni stupovi lipidne i energetske homeostaze, no njihova cjelovita uloga u mozgu nije još definirana i mogla bi biti od velikog značenja. Cilj ovog rada je sa sigurnošću identificirati lipidne kapljice kao aktivne sudionike upalnog procesa u mozgu tijekom Parkinsonove bolesti i ishemičnog moždanog udara koristeći modele 6-hidroksidopamina za Parkinsonovu bolest i uzorke sa trožilnom okluzijom za ishemični moždani udar. Također, identifikacija lipidnih kapljica kao aktivnih sudionika upalnih procesa u mozgu vrši se koristeći BV2 stanične culture tretirane s tapsigarginom.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.
Rad sadrži: 38 stranica, 17 grafičkih prikaza, 1 tablicu i 16 literaturnih navoda. Izvornik je na engleskom jeziku.
Ključne riječi: Parkinsonova bolest, moždani udar, mikroglija, miševi, štakori, stanična kultura, lipidne kapljice, upala
Mentor: Prof. Dr. sc. Lidija Bach-Rojecky, redovni profesor Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.
Komentor: Mikko Airavaara, Ph.D. Full Professor, University of Helsinki Faculty of Pharmacy
Ocjenjivači: Dr. sc. Lidija Bach-Rojecky, redovni profesor Sveučilišta u Zagrebu Farmaceutsko-biokemijskog
fakulteta.
Doc. Dr. sc. Sandra Šupraha Goreta, docentica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.
Prof. Dr. sc. Ana-Marija Domijan, izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.
Rad prihvaćen: rujan 2021.