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INSULIN RESISTANCE, ALZHEIMER'S DISEASE AND BRAIN WHITE MATTER INTEGRITY

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Insulin resistance is defined as an inability of target tissues to act in response to insulin, a crucial anabolic hormone. It is considered a prediabetic state and is often associated with obesity. Insulin resistance can be detected even years before the onset of type 2 diabetes, and thus it is an important target for prevention. Type 2 diabetes is one of the known preventable risk factors for Alzheimer's disease (AD), the most common type of memory disorder. Nevertheless, insulin resistance alone seems to be an independent risk factor for cognitive impairment and further development of AD. There are several possible pathophysiological explanations for this association. The purpose of this literature review is to investigate the possible connection between insulin resistance and loss of brain white matter integrity as both phenomena are known to occur in Alzheimer's disease.

In this literature review, the focus is on recent findings about insulin resistance and white matter integrity impairment detected by a modality of magnetic resonance imaging called diffusion tensor imaging (DTI). For background, insulin resistance, Alzheimer's disease and their connection are discussed in detail. To date, a few cross-sectional studies have been published that concentrate on insulin resistance and white matter integrity loss. These suggest that insulin resistance might have an impact on white matter integrity. More extensive follow-up studies are needed to establish a connection between insulin resistance and white matter integrity impairment.

Key words: insulin resistance, Alzheimer's disease, white matter integrity, diffusion tensor imaging

Table of contents

1 Introduction

1.1 Insulin resistance

1.1.1 Pathophysiology

Insulin is an essential anabolic hormone produced in the pancreas that regulates glucose uptake into the cells. Insulin resistance (IR) is a condition in which responsiveness to insulin has declined, meaning that insulin-mediated glucose uptake is impaired in tissues. Skeletal muscle is the most important tissue regarding glucose metabolism, but insulin also has effects on other tissues. In the liver, insulin resistance further stimulates gluconeogenesis. Compromised glucose uptake and upregulated gluconeogenesis together cause hyperglycemia. (Sesti, 2006; Laakso, 2015.) In adipose tissue insulin prevents lipolysis and release of free fatty acids into the circulation. It has been shown that free fatty acids further inhibit insulin signaling and promote hyperglycemia. (Gastaldelli et al., 2010.)

Inflammation (chronic low-grade inflammation) has been linked to the pathogenesis of IR as well. For instance, an inflammatory cytokine TNF-α (tumor necrosis factor alpha) is upregulated in adipose tissue and it contributes to IR (Hotamisligil et al., 1996; Verdile et al., 2015). Persistent IR burdens the β-cells of the pancreas and suppresses insulin production, eventually leading to insulin deficiency (Verdile et al., 2015).

Lifestyle and environmental factors contribute greatly to the development of IR, central obesity being perhaps the most significant factor. However, it has become clearer that genetics have an effect on IR as well. (Beale, 2013.) IR naturally predisposes to type 2 diabetes (T2D), but in addition it has strong associations with cardiovascular diseases. IR alters the lipid profile in blood: triglyceride level increases whereas high-density lipoprotein (HDL) cholesterol level decreases, which are both well-known atherosclerosis risk factors. Hypertension is often associated with IR, which may be explained by over-expression of renin-angiotensin-aldosterone-system. (Ormazabal et al., 2018.) The relationship between IR and cognitive impairment will be discussed in detail later in this work.

1.1.2 Measurements of insulin resistance

Clinically, the easiest measurement method for IR is the oral glucose tolerance test (OGTT) in which the subject ingests 75 g of glucose dissolved in water. Fasting plasma glucose has been measured before glucose intake and two hours after the test glucose value is measured again – or sometimes, for research purpose, various times over the two-hour period. In healthy adults, glucose value peaks at 30-60 minutes and returns to baseline at two hours. QUICKI (Quantitative Insulin sensitivity Check Index) describes insulin sensitivity only using fasting blood glucose and insulin values. (Borai, Livingstone and Ferns, 2007.)

The euglycemic hyperinsulinemic clamp is considered the golden standard of IR assessment but it is a rather invasive and complex test and used mostly in research. The method is based on infusing simultaneously both insulin and glucose into the study subject until a steady state of blood glucose is reached, and reliable values of exogenous insulin and glucose can be measured. (Borai, Livingstone and Ferns, 2007.) HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) is a mathematical model that can be used to estimate IR and β-cell function using plasma insulin and glucose values. It correlates well with the clamp test and is more practical to use. (Wallace, Levy and Matthews, 2004.)

1.1.3 Insulin in the brain

Glucose uptake into peripheral tissues is insulin dependent but the brain, however, utilizes glucose as its main energy source independently of insulin. Glucose transports through the blood-brain barrier via GLUT1 transporters and into neurons via GLUT3 transporters. (Benarroch, 2014.) Thus, it was long thought that insulin had virtually no effect on the brain. However, insulin receptors have been found to be expressed in all brain cell types although expression levels differ between regions (Arnold et al., 2018). Animal models have shown that insulin receptors are expressed most in olfactory bulb, hypothalamus, hippocampus, cortex and thalamus as well as in cerebellum, striatum, midbrain and brainstem (Arnold et al., 2018; Ferreira et al., 2018). According to present view, insulin is probably not produced in the central nervous system (CNS) but pancreatic insulin is rather transported through the blood-brain barrier (Kleinridders et al., 2014; Rhea, Salameh and Banks, 2019). Insulin level is significantly lower in cerebrospinal fluid than in blood, although their levels correlate with each other – which furthermore supports the pancreas as the source of central nervous system insulin

(Kleinridders et al., 2014). To summarize, brain glucose metabolism is not insulin dependent, but it appears to be insulin sensitive.

There is evidence suggesting that insulin in the brain influences the metabolism of the whole body by acting on hypothalamus: numerous animal studies have found that hypothalamic insulin administration reduces food intake. A similar effect is seen in humans as well: insulin administered intranasally or intracerebroventricularly acts in an anorexigenic manner, contrary to its peripheral effect. (Kullmann et al., 2016.) CNS insulin also suppresses gluconeogenesis and lipolysis (Arnold et al., 2018). Regarding cognition, insulin improves overall synaptic plasticity and memory. Insulin enhances long term potentiation (LTP) which is the one of the basic mechanisms of memory formation. It also affects catecholamine expression and might increase glucose usage in certain areas important for memory. (Cholerton, Baker and Craft, 2013.) One of the earliest experimental studies regarding CNS insulin and cognition in healthy young adults (N=38, age 18-34 years) found out that insulin administered intranasally improved delayed word recall up to 8 weeks after administration and even affected mood positively. (Benedict et al., 2004.)

To date, it has been somewhat controversial whether brain IR might be a phenomenon independent of peripheral IR. Responsiveness to insulin in brain cells has been found to be impaired in obese individuals as well as in individuals with T2D. In addition, age itself increases the risk of brain IR as the number of insulin receptors decrease. (Kullmann et al., 2016.) A hypothesis formed on the basis of several animal studies claims that dyslipidemia, common in diabetes and obesity, increases blood-brain barrier permeability which in turn leads to neuroinflammation, IR and neuronal degradation (Ferreira et al., 2018). IR has also been proposed to act on the blood-brain barrier permeability, meaning that peripheral IR impairs insulin transport into the brain (Heni et al., 2014). While the underlying mechanisms between cognitive impairment and brain IR remain uncertain, possible explanations are discussed later.

1.2 Alzheimer's disease

1.2.1 Epidemiology

Alzheimer's disease (AD) is the most common type of dementia worldwide (Lane, Hardy and Schott, 2018) and its prevalence is increasing rapidly as people are living longer than ever. According to the

World Health Organization, the amount of people suffering from dementia worldwide will almost triple in 35 years from 46 million to 131,5 million (Prince et al., 2015). In Finland, it has been estimated that there are approximately 100 000 people living with moderate to severe dementia and another 100 000 people with mild dementia today. AD alone constitutes 65-70 % of all dementia cases. (Ngandu and Kivipelto, 2018). The second most common type of dementia is vascular dementia which often overlaps with AD and shares common risk factors (Kivipelto, Mangialasche and Ngandu, 2018).

AD is a devastating progressive disease that gradually leads to death in approximately 8.5 years from diagnosis. First, a loss of episodic memory can be seen as well as psychiatric symptoms: depression, anxiety, aggression, and behavioral changes. Later on, motor functions deteriorate and eventually patients will be totally dependent of their caregivers as their ability to function declines. (Lane, Hardy and Schott, 2018.) Mild cognitive impairment, MCI, typically precedes AD diagnosis. MCI is a continuum of cognitive decline which does not necessarily lead to dementia, even though the incidence is 5 to 30 times higher than in a population with normal cognition. Risk factors for MCI conversing into AD are deep white matter lesions and ischemia, diabetes or prediabetes, psychiatric symptoms and APOEε4 genotype in addition to other genetic factors. (Campbell et al., 2013.)

Despite decades of research, there is so far neither a cure nor effective drugs to halt AD progression. Pathological changes often develop before any notable symptoms. Thus, it is important to be aware of modifiable risk factors in order to invest in prevention – preferably even decades before old age and possible disease onset.

1.2.2 Risk factors

Early-onset AD is a rare (<5% of all cases) hereditary form of AD that occurs in midlife, decades earlier than typically. There are three specific autosomal dominant genes known that cause the early-onset AD. (Bateman et al., 2011.) Regarding the more common sporadic late-onset AD, risk factors include both genetic and environmental factors. Different alleles of apolipoprotein E gene (APOE) significantly alter the risk of developing AD. APOE is a lipid transport protein expressed in the brain as well as in other tissues. APOE gene has three isoforms: APOE2, APOE3, APOE4 and alleles ε2, ε3, ε4. (Fan et al., 2019.) APOEε4 genotype is claimed to be the most significant risk factor for sporadic AD (Lane, Hardy and Schott, 2018). Being a homozygotic APOEε4 carrier increases the

risk of AD to 12-fold compared to non-carriers (Corder et al., 1993). Other genetic risk factors have been identified recently but their effect seems to be minimal compared to APOE4 genotypes (Karch and Goate, 2015). Apart from APOE genotype, non-modifiable risk factors include age and previous head injuries (Ballard et al., 2011). Aging increases incidence of AD exponentially: the incidence of AD is 1.6 % in the age group 60-65 years but almost 40 % among people over 90 (Ngandu and Kivipelto, 2018).

Barnes and Yaffe estimated that seven, potentially modifiable, risk factors attribute to around half of all AD cases. These include diabetes, hypertension, obesity, smoking, depression and low educational level or cognitive inactivity. (Barnes and Yaffe, 2011.) In addition, hypercholesterolemia seems to be a considerable independent risk factor for AD (Xue-shan et al., 2016.) as well as cerebrovascular diseases such as stroke or microvascular lesions (Ngandu and Kivipelto, 2018). IR as a risk factor for AD will be discussed later.

Protective factors against AD include cognitive reserve: both high educational level and leisure activity can be protective. A diet high in unsaturated fats and antioxidants is favorable as well as physical activity. There are controversial results concerning vitamin supplementations or estrogen as a hormone replacement therapy. (Ballard et al., 2011; Silva et al., 2019.)

1.2.3 Pathophysiology

The hallmark pathological features of AD are extracellular amyloid plaques (also called neurite or senile plaques) and intraneuronal neurofibrillary tangles. Amyloid plaques consist of beta-amyloid peptides (Aβ40 and Aβ42 isoforms), which are cleavage products of amyloid precursor protein. In normal conditions Aβ peptides are soluble monomers but in higher levels they form oligomers and eventually fibrils into amyloid plaques. Neurofibrillary tangles contain misfolded and hyperphosphorylated tau, a microtubule-associated protein. (Serrano-Pozo et al., 2011.)

The so-called amyloid hypothesis suggests an imbalance between Aβ clearance and production activates the development of Alzheimer's disease. Nevertheless, the number of plaques does not seem to correlate with clinical symptoms and beta-amyloid deposits can be seen decades before any cognitive decline. Aβ42 oligomers have nevertheless been shown to impair synaptic function and structure, stimulate tau hyperphosphorylation and in addition activate inflammation processes.

(Selkoe and Hardy, 2016.) Contrary to amyloid plaques, the amount of neurofibrillary tangles correlates with clinical symptoms in AD (Serrano-Pozo et al., 2011). However, tau pathology is seen also in frontotemporal dementia independently of amyloid accumulation. (Small and Duff, 2008.) Braak staging divides neurofibrillary tangle distribution and thus disease progression into six stages according to anatomical distribution: I-II transentorhinal, III-IV limbic and V-VI isocortical stages (Braak et al., 2006).

Another pathological mechanism in AD is neuroinflammation, which has been suggested to act as a mediator between tau and amyloid accumulation. Microglia and astrocytes, immune cells in the brain, typically have anti-inflammatory effects in the early stages of AD while neuroprotective pathways are working. Nevertheless, overexpressed in AD they begin to secrete inflammatory cytokines which results in neuronal degradation. (Kinney et al., 2018.)

Acetylcholine is one of the most important neurotransmitters and cholinergic neurons are widely distributed in the brain. Acetylcholine plays a role in many essential neuronal functions, for example sleep cycle, memory, and attention. Cholinergic neurons in the basal forebrain are severely damaged in AD and the loss of cholinergic neurons correlates with typical symptoms. There are drugs currently in use that are targeted to acetylcholine loss, but they only alleviate the symptoms of AD, and do not halt disease progression. Cholinesterase inhibitors block the breakdown of the neurotransmitter and thus increase the amount of acetylcholine for a short period of time. (Ferreira-Vieira et al., 2016.)

1.2.4 Diagnostics

At present, AD diagnosis can only be determined as “probable” during life since only the postmortem neuropathological diagnosis is considered certain. Nevertheless, several diagnostics tools have been studied recently and diagnostic criteria might be changing in the future. (Sorbi et al., 2012.) AD is rather difficult to diagnose clinically. Careful history of the symptoms must be taken from both the patient and the patient’s partner, other family member or caregiver. (Ballard et al., 2011.) MMSE (mini mental state examination) is a widely used 30-question screening test for dementia, but it alone has low sensitivity and specificity for AD (Arevalo-Rodriguez et al., 2015). CERAD (The Consortium to Establish a Registry for Alzheimer's Disease) is a more comprehensive test of cognitive function and memory that is used in Finland for screening for memory disorders since its sensitivity for mild cognitive impairment is better (Hänninen et al., 2010).

In practice, magnetic resonance imaging (MRI) is often used to refine clinical AD diagnosis. It can also exclude secondary causes of dementia, such as tumors or inflammatory diseases. (Sorbi et al., 2012.) Structural MRI reveals brain atrophy that at first in AD is seen to occur in medial temporal lobes. Hippocampus, an essential brain region for memory, is a part of the medial temporal lobe and its atrophy correlates to memory loss and other cognitive deficits in patients with AD. In addition to hippocampal atrophy, other parts of the limbic system are affected, and along disease progression also broader cortical regions begin to degenerate. (Chandra et al., 2019.) Traditionally, AD was considered a gray matter disease, but this may not be the whole truth, since white matter abnormalities can be seen with structural MRI in AD (Caso, Agosta and Filippi, 2016). Diffusion tensor imaging, a modality of MRI, detects white matter abnormalities more specifically and will be discussed later.

Positron emission tomography (PET) is rarely used in the clinical diagnosis of AD, but it is a useful tool in research. However, PET imaging is used in the diagnosis of memory impairment for working-age patients when the diagnosis remains unclear after MRI imaging and cerebrospinal fluid samples. (Memory Disorders: Current Care Guidelines, 2020.) PET imaging utilizes radioligands that express changes in the brain metabolism, circulation, or specific protein aggregations, depending on the used ligand, and the results are often combined with structural MRI imaging. Fluorodeoxyglucose (FDG) is a glucose analog ligand that in AD shows hypometabolism first in the precuneus and posterior cingulate cortex and later in temporo-parietal regions (Valotassiou et al., 2018). Amyloid burden is seen to reversely correlate with glucose metabolism (Bao et al., 2017). The most extensively studied radioligand regarding AD, ^{11}C-Pittsburgh Compound B (PiB), expresses amyloid deposition. It has a very high sensitivity of 96 % for AD but a specificity of only 76 %. Most patients with MCI show PiB-retention in a PET scan as well. (Marcus, Mena and Subramaniam, 2014.) Since the amount of tau-consisting neurofibrillary tangles seems to correlate with clinical symptoms, tau PET markers have been studied but none of them are in clinical use thus far. (Bao et al., 2017; Valotassiou et al., 2018).

Cerebrospinal fluid (CSF) biomarkers are used in differential diagnostics of AD as well as in research. There are three CSF biomarkers in use at present: amyloid-$\beta_{1\text{-}42}$, phosphorylated tau (P-tau) and total tau (T-tau) (Blennow and Zetterberg, 2013). Typical CSF marker findings in AD are high level of total and phosphorylated tau and low level of amyloid-$\beta_{1\text{-}42}$ which correlate with biopsy findings. (Seppälä et al., 2012; Skillbäck et al., 2015). This notion is in line with the idea of amyloid aggregation in the brain and a decrease of amyloid-$\beta_{1\text{-}42}$ has been seen also in people with MCI. T-

tau on the other hand seems to predict disease progression rapidity, while P-tau correlates with the amount of neurofibrillary tangles. (Zetterberg, Rohrer and Schott, 2018.)

1.3 Insulin resistance and Alzheimer's disease

IR is a shared feature of T2D, obesity, and AD. The link between IR and AD has been studied with great interest using animal models and medical imaging such as PET. (Kullmann et al., 2016; Diehl, Mullins and Kapogiannis, 2017.) In a follow-up study of 15 years, IR in midlife was associated with a higher amount of brain amyloid accumulation in PET scans in non-demented individuals (Ekblad et al., 2018), while opposed PET scan results have also been found (Thambisetty et al., 2013). AD has even been proposed to be "type 3 diabetes" because of the notion that AD pathology contributes to glucose metabolism disruption (Steen et al., 2005). However, this notion is somewhat controversial. Because both IR and AD are gradually developing conditions and share overlapping risk factors, their causality may be difficult to establish. Furthermore, the association between peripheral and brain IR remains unclear. (Arnold et al., 2018; Ferreira et al., 2018.)

The association between cognitive impairment and IR has been mostly studied in middle-aged and elderly populations. Age itself increases risk for both conditions so this connection may be coincidental. (Arnold et al., 2018.) However, in the Framingham Heart Study, a wide cohort study, diabetes was associated with impaired memory and attention function (n = 2,126) in young and middle-aged people (mean age 40 ± 9 years). Diabetic subjects and subjects with elevated fasting blood glucose exhibited both decreased gray matter volumes and fractional anisotropy assessed by MRI (n = 1,597). (Weinstein et al., 2015.)

Beta-amyloid and insulin share a common catalytic enzyme IDE, insulin degrading enzyme. It has been observed that in a hyperinsulinemic state, Aβ cleavage is reduced, which could be a possible link between IR and AD pathology. (Stanley, Macauley and Holtzman, 2016.) In animal studies, intracerebroventricular injection of Aβ-oligomers has induced neuronal IR and even peripheral IR. Another common feature of diabetes and AD is the formation of advanced glycation end products, that show in hyperglycemic conditions – and it might be yet another reason for blood-brain barrier disintegration and even neurodegeneration. (Ferreira et al., 2018.)

Even though it is not known precisely how T2D and AD are linked, diabetes drugs have been studied in the treatment of Alzheimer's disease. Insulin administered subcutaneously mainly acts on peripheral tissues, but insulin administered nasally bypasses the blood-brain barrier and reaches the CNS trough the cribriform plate. Nasal insulin has been studied in both healthy subjects and MCI or AD patients. Even in cognitively healthy subjects, word recall was improved after regular insulin administration which further emphasizes its role in normal cognition. (Yarchoan and Arnold, 2014.) A systematic review from 2018 concluded that in seven distinct studies (n = 293), insulin administered intranasally improved word and/or story recall in non-APOE4-carrier AD or MCI patients (Avgerinos et al., 2018).

1.4 Diffusion tensor imaging and white matter integrity

1.4.1 Principles of diffusion tensor imaging

Diffusion tensor imaging (DTI) is a fairly new modality of MRI that is increasingly used in research and clinically. DTI provides information on brain white matter tract anatomy noninvasively *in vivo* based on water diffusion values. Water molecules tend to move randomly due to heat and this movement can be observed even in a postmortem brain – which means that it is not solely a physiological phenomenon. DTI characterizes diffusion three-dimensionally which allows for a wide range of applications. (Mori and Zhang, 2006; Mukherjee et al., 2008.)

Fractional anisotropy (FA) is perhaps the most used value in DTI. It describes symmetry of diffusion values. Diffusion in neurons is naturally faster along axons than perpendicularly which allows for fiber orientation to be assessed by diffusion values. Thus, FA is considered a generalization of white matter integrity. (Bihan et al., 2001; Mori and Zhang, 2006.) FA and mean diffusivity (MD; also apparent diffusion coefficient, ADC) are both derived from so-called eigenvectors and their eigenvalues $\lambda 1$, $\lambda 2$, and $\lambda 3$. Primary eigenvalue $\lambda 1$ describes both the magnitude and direction of greatest water diffusion along fibers, whereas the other eigenvalues indicate diffusion transversally and their mean value is called radial diffusivity (RD). FA value varies from 0 to 1: the maximum value 1 indicating that only linear diffusion occurs along primary eigenvector and diffusion is isotropic. (Mukherjee et al., 2008.)

Tract-based spatial statistics (TBSS) is a DTI data analysis method developed to improve cross-subject studies. In order to assess regional changes between subjects, FA data from subjects is first registered non-linearly and aligned together, which in TBSS is done by creating a "group mean FA skeleton" which ideally represents common tracts among subjects. TBSS reduces the need for smoothing and enables a more trustworthy voxelwise statistical analysis. (Smith et al., 2006.) Tractography, as per its name, is a method of visualizing white matter tracts reconstructed from DTI data. Tract orientations are coded in specific colors to make a tractogram demonstrative. Simply put, a tractogram, a tractographic image, can be derived from above-mentioned diffusion values when it is assumed that one fiber orientation is predominant in a single three-dimensional voxel. These presumptions of homogeneity naturally lead to simplifications and false positives as well as false negatives. Overall, it is a method that requires a careful user. (Jeurissen et al., 2019.)

1.4.2. White matter integrity in Alzheimer's disease

As stated before, AD affects white matter of the brain in addition to gray matter. According to current knowledge, at least some of the changes may be independent of gray matter atrophy. Mechanisms of white matter damage in AD remain unclarified and there are different theories of its origin as to whether white matter damage is independent of gray matter damage or not. The so-called Wallerian degeneration means axonal disruption when separated from cell bodies, thus linking white matter damage to gray matter damage. However, there are some studies that suggest white matter damage precede grey matter damage in AD. (Sachdev et al., 2013; Caso, Agosta and Filippi, 2016.) According to the retrogenesis model, 'reverse myelogenesis' is the mechanism of white matter damage. It suggests white matter as the primary source of AD pathogenesis, but this theory remains somewhat contradictory and may be linked to underlying cerebrovascular conditions. Neuroinflammation has also been suggested to be one mechanism of WM damage. (Mayo et al., 2017.)

White matter hyperintensity seen in conventional MRI indicates demyelination and axonal loss along white matter tracts in AD. DTI nonetheless provides more specific information on exact WM tracts affected by AD. Tractography studies have shown wide white matter damage in major limbic tracts including cingulum and fornix and corticocortical association tracts such as corpus callosum in AD patients. (Caso, Agosta and Filippi, 2016.)

In a cross-sectional study from 2011, DTI was performed on 23 AD patients, 15 patients with amnestic mild cognitive impairment (aMCI) and 15 control subjects. Researchers used two most common DTI values MD and FA to estimate white matter microstructure impairment. In addition, gray matter morphometry was used to investigate whether WM and GM atrophy correlated. AD patients exhibited increase in MD widely across various WM tracts: e.g., cortico-cortical, corticospinal, interhemispheric, and limbic tracts while FA decrease was seen only in smaller tracts. There was an increase in axial diffusivity in aMCI patients, mostly in tracts projecting to the frontal cortex but no significant changes in other values were found. In AD patients some correlations between WM impairment and regional gray matter atrophy were found but rather inconsistently. (Agosta et al., 2011.)

In a more recent longitudinal study by Mayo et al. (2017), DTI data obtained from 34 AD patients and 33 controls were compared at baseline and at one year. In TBSS analysis, AD patients showed increased MD and decreased FA widely in white matter tracts in the medial temporal lobe compared to control subjects, and these changes were greater at one year. Also, healthy controls showed signs of white matter microstructure impairment due to aging but less extensively than AD patients, and in controls hippocampal cingulum was spared unlike in AD. These findings appeared to be in line with neuropathological progression in AD. (Mayo et al., 2017.)

2 Methods

This literature review was done in Turku PET center as a part of research project CIRI (Cognition, Insulin Resistance, and Inflammation), a neuroimaging study on 60 elderly volunteers which was conducted to evaluate the associations between midlife IR and late-life cerebral changes related to cognitive decline. The study participants underwent magnetic resonance imaging including DTI imaging, and positron emission tomography with two different radiotracers to assess brain amyloid burden and neuroinflammation. In addition, a comprehensive cognitive test battery was performed to all participants.

Database search was performed on the 30th of November in 2019 in PubMed database. Following search query was used: (DTI* OR "diffusion tensor imaging" OR tractograph*) AND (insulin resistance* OR hyperglycemia* OR diabetes* OR metabolic syndrome* OR MetS*) AND (cogniti* OR MCI* OR AD* OR Alzheimer's disease* OR dementia*). Only articles written in English and original research articles were included.

For the literature review, there were following criteria for included articles: 1) study includes an insulin resistant (not only a T2D group) and control group 2) white matter changes were assessed using DTI 3) cognitive performance was measured 4) subjects had no dementia. 149 articles were found using the before-mentioned search query. Only three of those met the inclusion criteria and were included in this literature review.

3 Review of the literature

The purpose of this literature review is to evaluate the association between IR and white matter integrity according to current knowledge. In addition, a possible link between AD and the action of IR on brain white matter is considered.

While IR as a risk factor for white matter abnormalities is not established, more is known about the effects of diabetes on white matter tracts. In a study by Reijmer et al. (2013), a group of elderly (mean age 71 ± 5 years) T2D patients demonstrated abnormalities widely in white matter tracts using tractography. These impairments were associated with poorer cognitive function in the diabetic group, although the same association was not seen in control group. In the T2D group, MD was significantly increased in all major white tracts studied (the superior longitudinal fasciculus, the uncinate fasciculus, the inferior longitudinal fasciculus, and the genu and splenium of the corpus callosum).

Other studies have found similar results regarding the effects of diabetes on white matter studying middle-aged patients (Tan et al., 2016) and they might be associated with microvascular complications of diabetes (Zhuo et al., 2019). Xiong et al. (2016) suggest that a change in DTI values might indicate a cognitive decline in patients with T2D. In their study, diabetic subjects were divided

into those with MCI and without. TBSS analysis revealed that those with MCI exhibited greater MD and FA changes than those without compared to healthy controls. However, only cross-sectional results were reported. (Xiong et al., 2016.)

A recent study found out that heightened blood glucose levels, even below diagnostic cutoff values for diabetes, in healthy young adults (mean age 28.8 years, n = 1206) were associated with poorer white matter integrity. Glycated hemoglobin HbA1c (range 4.1 - 6.3 %) was found to have a negative correlation with both cognitive performance and FA value. (Repple et al., 2019)

A few studies have been focusing specifically on IR and white matter abnormalities and these are the focus of this review. The first one by Ryu et al. (2014) included 127 healthy middle-aged and older participants that were divided into two subgroups according to their IR status assessed by HOMA-IR. DTI was performed on all subjects and analyzed using TBSS. High HOMA-IR group (n = 27) showed lower axial diffusivity (DA) values broadly in white matter regions including frontal, temporal and parietal white matter, corpus callosum and corona radiata (see Fig. 1). FA changes were not as significant but overlapping of both FA and DA decrease was seen in corpus callosum and some connection fibers. APOE status did not have an effect on these findings. White matter integrity loss was associated with IR independently of aging and usage of antihypertensive medication in this study. Cognitive function was assessed by MMSE and there was virtually no difference between the study groups.

More recently, Liang et al. (2019) published a TBSS study with somewhat similar results. Sixty middle-aged participants were divided into prediabetic and control groups. Their IR status was assessed by OGTT. The APOE status of the participants was not identified. In the prediabetic group FA was lower in right corpus callosum and left and right superior longitudinal fasciculus compared to the control group. No other differences were observed between groups in other DTI indices: MD, RD, and DA. In addition to MMSE, other neuropsychological tests, MoCA, SAS and SDS, were used. FA value correlated weakly with neuropsychological test results, but these findings were not statistically significant.

The Maastricht Study is a large cohort study focused on T2D and its comorbidities. Vergoossen et al. (2019) published a tractography study (N=2219) about white matter connectivity in both T2D and prediabetes. Whole brain tractography was performed on three subgroups: subjects with normal glucose metabolism, prediabetes, and T2D. Potential confounding variables, such as sex, education

level and cardiovascular risk factors were considered, and analyses adjusted for them. Overall node degree, indicating WM area connectivity, was found to be significantly lower in both prediabetic and diabetic groups compared to control group. This decrease of node degree equaled to 2.3 and 10.4 years of aging, respectively, compared to controls with normal glucose metabolism. Furthermore, even hyperglycemia continuously measured by HbA1c and fasting blood glucose was inversely correlated to node degree.

In prediabetic subjects WM tract volume was diminished only in interhemispheric connections while in T2D both intra- and interhemispheric tracts were affected. Indices describing network organization, so called graph measures, were affected in prediabetes. Local connectivity assessed by clustering coefficient was lower in addition to lower local efficiency. These changes were not observed in diabetic group, but instead T2D associated with higher communicability which was hypothesized to be due to adaptation to white matter lesions. MMSE results were marginally lower in both prediabetic and diabetic groups and in this large cohort the difference was found to be statistically significant. (Vergoossen et al., 2019.)

Table 1. A comparison of demographic data and results between DTI studies.

Reference	N	IR test	Age (IR/control)	MMSE (IR/control)	DTI analysis method	DTI results
Liang et al., 2019	60 (30 prediabetic, 30 controls)	OGTT	55.0 ± 6.7 / 52.8 ± 7.5	29.4 ± 0.7 / 29.7 ± 0.5 (*p* 0.14)	TBSS	decreased FA in prediabetic group in right part of CC and left superior longitudinal fasciculus
Ryu et al., 2014	127 (27 high HOMA-IR, 100 controls)	HOMA-IR	62.5 ± 11.5 / 62.1 ± 10.3	28.6 ± 1.5 / 28.6 ± 1.5 (*p* 0.89)	TBSS	decreased FA in CC, parts of corona radiata; decreased DA in WM of frontal, parietal and temporal lobes
Vergoossen et al., 2019	2219 (510 with T2D, 348 prediabetic, 1,361 controls)	OGTT	62.5 ± 7.6, 61.2 ± 7.5 / 57.6 ± 8.1	28.7 ± 1.3, 28.9 ± 1.1 / 29.2 ± 1.1 (*p* <0.0001)	whole brain tractography	both prediabetes and T2D groups exhibit lower node degree and WM tract volume

OGTT= oral glucose tolerance test, TBSS= tract based spatial statistics, FA= fractional anisotropy, DA= axial diffusivity, CC=corpus callosum, HOMA-IR=homeostasis model assessment of insulin resistance.

4 Discussion

In this literature review, IR and its effect on brain white matter integrity was investigated. Three publications from recent years addressing the topic were included. In all the included studies DTI showed changes in diffusion values and connectivity and thus white matter microstructure in subjects with IR. Cognitive performance assessed by MMSE showed minimal impairment in IR only in one study. MMSE alone in non-demented individuals is not a sensitive tool to assess cognitive performance (Arevalo-Rodriguez et al., 2015) and in order to study the relationship between IR, white matter impairment and cognitive performance, more extensive neuropsychological tests are needed in future studies.

The two TBSS studies had both relatively small study populations and were cross-sectional (Ryu et al., 2014; Liang et al., 2019) while the study by Veergoossen et al. included a significantly larger study population. The study populations of all studies were roughly of same age (mean ages 52.8 – 62.5 years) but from different cultural backgrounds and supposedly different educational systems and different levels of education.

In the Maastricht study the differences in MMSE scores between diabetic and non-diabetic groups were found to be statistically significant. However, education levels varied substantially between the groups: the diabetic and prediabetic groups had a lower education level in average compared to the non-diabetic group but this was taken into account in the analyses. (Vergoossen et al., 2019.) High educational level is a known protective factor against cognitive decline and AD (Ballard et al., 2011). In diabetic and prediabetic groups, there were more metabolic risk factors present: higher body mass index, higher prevalence of hypertension and lipid-modifying-medication, which were taken into account – contrary to the other studies here (Vergoossen et al., 2019).

Regarding the methods to assess IR, Ryu et al. used HOMA-IR assessment while the others used a simpler method, OGTT. High HOMA-IR group in their study did not equal prediabetic, because both groups even had some participants diagnosed with T2D and users of oral diabetes medication (Ryu et al., 2014) and thus the results of this study cannot be generalized for prediabetes.

Furthermore, there were two distinct DTI analysis methods used in these studies, tractography and TBSS, which makes it difficult to compare white matter impairment in any specific brain regions between the studies. In addition, TBSS studies both exhibited changes in diffusion values, but not in the same parameters. This demonstrates the need for further studies. While AD has been shown to affect brain white matter integrity in various regions, it is not yet possible to draw conclusions that these changes are mediated by IR as there is not enough evidence to support this hypothesis.

In conclusion, the results of this literature review suggest that IR in middle-aged subjects might contribute to brain white matter integrity loss. IR, prediabetes, and type 1 and 2 diabetes have consistently been linked to cognitive decline, but the underlying pathophysiological mechanisms causing cognitive decline are not yet clear. Based on the existing literature, white matter integrity loss seems to play a part in cognitive decline in prediabetic individuals. Nevertheless, follow-up studies need to be conducted in the future to establish this connection and a possible further connection to the development of AD.

References

Agosta, F. *et al.* (2011) 'White matter damage in Alzheimer disease and its relationship to gray matter atrophy', *Radiology*, 258(3), pp. 853–863.

Arevalo-Rodriguez, I. *et al.* (2015) 'Mini-Mental State Examination (MMSE) for the detection of Alzheimer's disease and other dementias in people with mild cognitive impairment (MCI)', *Cochrane Database of Systematic Reviews*, (3), p. CD010783.

Arnold, S. E. *et al.* (2018) 'Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums', *Nature Reviews Neurology*. Nature Publishing Group, 14(3), pp. 168–181.

Avgerinos, K. I. *et al.* (2018) 'Intranasal insulin in Alzheimer's dementia or mild cognitive impairment: a systematic review.', *Journal of neurology*. NIH Public Access, 265(7), pp. 1497–1510.

Ballard, C. *et al.* (2011) 'Alzheimer's disease.', *Lancet (London, England)*, 377(9770), pp. 1019–31.

Bao, W. *et al.* (2017) 'PET Imaging for Early Detection of Alzheimer's Disease: From Pathologic to Physiologic Biomarkers', *PET Clinics*. Elsevier, 12(3), pp. 329–350.

Barnes, D. E. and Yaffe, K. (2011) 'The projected effect of risk factor reduction on Alzheimer's disease prevalence', *The Lancet Neurology*, 10(9), pp. 819–828.

Bateman, R. J. *et al.* (2011) 'Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease.', *Alzheimer's research & therapy*, 3(1), p. 1.

Beale, E. G. (2013) 'Insulin Signaling And Insulin Resistance', *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*. NIH Public Access, 61(1), p. 11.

Benarroch, E. E. (2014) 'Brain glucose transporters: implications for neurologic disease.', *Neurology*, 82(15), pp. 1374–9.

Benedict, C. *et al.* (2004) 'Intranasal insulin improves memory in humans', *Psychoneuroendocrinology*, 29(10), pp. 1326–1334.

Bihan, D. Le *et al.* (2001) 'Diffusion tensor imaging: Concepts and applications', *Journal of Magnetic Resonance Imaging*. John Wiley & Sons, Ltd, 13(4), pp. 534–546.

Blennow, K. and Zetterberg, H. (2013) 'The Application of Cerebrospinal Fluid Biomarkers in Early Diagnosis of Alzheimer Disease', *Medical Clinics of North America*. Elsevier, 97(3), pp. 369–376.

Borai, A., Livingstone, C. and Ferns, G. A. A. (2007) *The biochemical assessment of insulin resistance*, *Ann Clin Biochem*.

Braak, H. *et al.* (2006) 'Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry', *Acta Neuropathologica*. Springer-Verlag, 112(4), pp. 389–404.

Campbell, N. L. *et al.* (2013) 'Risk factors for the progression of mild cognitive impairment to dementia', *Clinics in Geriatric Medicine*, pp. 873–893.

Caso, F., Agosta, F. and Filippi, M. (2016) 'Insights into White Matter Damage in Alzheimer's Disease: From Postmortem to in vivo Diffusion Tensor MRI Studies.', *Neuro-degenerative diseases*, 16(1–2), pp. 26–33.

Chandra, A. *et al.* (2019) 'Magnetic resonance imaging in Alzheimer's disease and mild cognitive impairment', *Journal of Neurology*, 266(6), pp. 1293–1302.

Cholerton, B., Baker, L. D. and Craft, S. (2013) 'Insulin, cognition, and dementia', *European Journal of Pharmacology*, 719(1–3), pp. 170–179.

Corder, E. H. *et al.* (1993) 'Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families.', *Science (New York, N.Y.)*, 261(5123), pp. 921–3.

Diehl, T., Mullins, R. and Kapogiannis, D. (2017) 'Insulin resistance in Alzheimer's disease.', *Translational research : the journal of laboratory and clinical medicine*. NIH Public Access, 183, pp. 26–40.

Ekblad, L. L. *et al.* (2018) 'Midlife insulin resistance, APOE genotype, and late-life brain amyloid accumulation', *Neurology*. Lippincott Williams and Wilkins, 90(13), pp. e1150–e1157.

Fan, J. *et al.* (2019) 'The Contribution of Genetic Factors to Cognitive Impairment and Dementia: Apolipoprotein E Gene, Gene Interactions, and Polygenic Risk.', *International journal of molecular sciences*, 20(5), p. 1177.

Ferreira-Vieira, T. H. *et al.* (2016) 'Alzheimer's disease: Targeting the Cholinergic System.', *Current neuropharmacology*, 14(1), pp. 101–15.

Ferreira, L. S. S. *et al.* (2018) 'Insulin Resistance in Alzheimer's Disease', *Frontiers in Neuroscience*, 12, p. 830.

Gastaldelli, A. *et al.* (2010) 'Insulin resistance, adipose depots and gut: interactions and pathological implications.', *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver*, 42(5), pp. 310–9.

Hänninen, T. *et al.* (2010) '[Early detection of cognitive changes in memory diseases: new cut-off scores for the Finnish version of CERAD neuropsychological battery].', *Duodecim; laaketieteellinen aikakauskirja*, 126(17), pp. 2013–21.

Heni, M. *et al.* (2014) 'Evidence for altered transport of insulin across the blood–brain barrier in insulin-resistant humans', *Acta Diabetologica*. Springer Milan, 51(4), pp. 679–681.

Hotamisligil, G. S. *et al.* (1996) 'IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance.', *Science (New York, N.Y.)*, 271(5249), pp. 665–8.

Jeurissen, B. *et al.* (2019) 'Diffusion MRI fiber tractography of the brain', *NMR in Biomedicine*. John Wiley and Sons Ltd.

Karch, C. M. and Goate, A. M. (2015) 'Alzheimer's Disease Risk Genes and Mechanisms of Disease Pathogenesis', *Biological Psychiatry*, 77(1), pp. 43–51.

Kinney, J. W. *et al.* (2018) 'Inflammation as a central mechanism in Alzheimer's disease', *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, 4, pp. 575–590.

Kivipelto, M., Mangialasche, F. and Ngandu, T. (2018) 'Lifestyle interventions to prevent cognitive impairment, dementia and Alzheimer disease', *Nature Reviews Neurology*. Nature Publishing Group, 14(11), pp. 653–666.

Kleinridders, A. *et al.* (2014) 'Insulin action in brain regulates systemic metabolism and brain function.', *Diabetes*. American Diabetes Association, 63(7), pp. 2232–43.

Kullmann, S. *et al.* (2016) 'Brain Insulin Resistance at the Crossroads of Metabolic and Cognitive Disorders in Humans', *Physiological Reviews*, 96(4), pp. 1169–1209.

Laakso, M. (2015) 'Is Insulin Resistance a Feature of or a Primary Risk Factor for Cardiovascular Disease?', *Current Diabetes Reports*, 15(12), p. 105.

Lane, C. A., Hardy, J. and Schott, J. M. (2018) 'Alzheimer's disease', *European Journal of Neurology*, 25(1), pp. 59–70.

Liang, M. *et al.* (2019) 'Diffusion tensor imaging of white matter in patients with prediabetes by trace-based spatial statistics', *Journal of Magnetic Resonance Imaging*. John Wiley & Sons, Ltd, 49(4), pp. 1105–1112.

Marcus, C., Mena, E. and Subramaniam, R. M. (2014) 'Brain PET in the Diagnosis of Alzheimer's Disease', *Clinical Nuclear Medicine*, 39(10), pp. e413–e426.

Mayo, C. D. *et al.* (2017) 'Longitudinal changes in microstructural white matter metrics in Alzheimer's disease.', *NeuroImage. Clinical*. Elsevier, 13, pp. 330–338.

Mori, S. and Zhang, J. (2006) 'Principles of Diffusion Tensor Imaging and Its Applications to Basic Neuroscience Research', *Neuron*. Cell Press, 51(5), pp. 527–539.

Mukherjee, P. *et al.* (2008) 'Diffusion Tensor MR Imaging and Fiber Tractography: Theoretic Underpinnings', *American Journal of Neuroradiology*, 29(4), pp. 632–641.

Ngandu, T. and Kivipelto, M. (2018) 'Multidomain lifestyle interventions in the prevention of memory disorder epidemic', *Duodecim; laaketieteellinen aikakauskirja*, 134(24), pp. 2547–53.

Ormazabal, V. *et al.* (2018) 'Association between insulin resistance and the development of cardiovascular

disease', *Cardiovascular Diabetology*, 17(1), p. 122.
Prince, M. *et al.* (2015) *World Alzheimer Report 2015 The Global Impact of Dementia An Analysis of Prevalence, Incidence, Cost and Trends*.
Repple, J. *et al.* (2019) 'Variation of HbA1c affects cognition and white matter microstructure in healthy, young adults', *Molecular Psychiatry*. Nature Publishing Group.
Rhea, E. M., Salameh, T. S. and Banks, W. A. (2019) 'Routes for the delivery of insulin to the central nervous system: A comparative review', *Experimental Neurology*. Academic Press, 313, pp. 10–15.
Ryu, S. Y. *et al.* (2014) 'Effects of insulin resistance on white matter microstructure in middle-aged and older adults', *Neurology*. Lippincott Williams and Wilkins, 82(21), pp. 1862–1870.
Sachdev, P. S. *et al.* (2013) 'Is Alzheimer's a disease of the white matter?', *Current Opinion in Psychiatry*, 26(3), pp. 244–251.
Selkoe, D. J. and Hardy, J. (2016) 'The amyloid hypothesis of Alzheimer's disease at 25 years', *EMBO Molecular Medicine*, 8(6), pp. 595–608.
Seppälä, T. T. *et al.* (2012) 'CSF biomarkers for Alzheimer disease correlate with cortical brain biopsy findings.', *Neurology*. Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology, 78(20), pp. 1568–75.
Serrano-Pozo, A. *et al.* (2011) 'Neuropathological Alterations in Alzheimer Disease', *Cold Spring Harbor Perspectives in Medicine*, 1(1), pp. a006189–a006189.
Sesti, G. (2006) 'Pathophysiology of insulin resistance.', *Best practice & research. Clinical endocrinology & metabolism*, 20(4), pp. 665–79.
Silva, M. V. F. *et al.* (2019) 'Alzheimer's disease: risk factors and potentially protective measures', *Journal of Biomedical Science*, 26(1), p. 33.
Skillbäck, T. *et al.* (2015) 'Cerebrospinal fluid tau and amyloid-β1-42 in patients with dementia', *Brain*. Narnia, 138(9), pp. 2716–2731.
Small, S. A. and Duff, K. (2008) 'Linking Abeta and tau in late-onset Alzheimer's disease: a dual pathway hypothesis.', *Neuron*, 60(4), pp. 534–42.
Smith, S. M. *et al.* (2006) 'Tract-based spatial statistics: Voxelwise analysis of multi-subject diffusion data', *NeuroImage*. Academic Press, 31(4), pp. 1487–1505.
Society, W. group set up by the F. M. *et al.* (2020) *Memory Disorders. Current Care Guidelines.*
Sorbi, S. *et al.* (2012) 'EFNS-ENS Guidelines on the diagnosis and management of disorders associated with dementia', *European Journal of Neurology*, 19(9), pp. 1159–1179.
Stanley, M., Macauley, S. L. and Holtzman, D. M. (2016) 'Changes in insulin and insulin signaling in Alzheimer's disease: cause or consequence?', *The Journal of experimental medicine*. The Rockefeller University Press, 213(8), pp. 1375–85.
Steen, E. *et al.* (2005) 'Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?', *Journal of Alzheimer's disease : JAD*, 7(1), pp. 63–80.
Tan, X. *et al.* (2016) 'Micro-structural white matter abnormalities in type 2 diabetic patients: a DTI study using TBSS analysis', *Neuroradiology*. Springer Verlag, 58(12), pp. 1209–1216.
Thambisetty, M. *et al.* (2013) 'Glucose intolerance, insulin resistance, and pathological features of Alzheimer disease in the Baltimore longitudinal study of aging', *JAMA Neurology*. American Medical Association, 70(9), pp. 1167–1172.
Valotassiou, V. *et al.* (2018) 'SPECT and PET imaging in Alzheimer's disease', *Annals of Nuclear Medicine*. Springer Japan, 32(9), pp. 583–593.
Verdile, G. *et al.* (2015) 'Inflammation and Oxidative Stress: The Molecular Connectivity between Insulin Resistance, Obesity, and Alzheimer's Disease', *Mediators of Inflammation*. Hindawi Publishing Corporation, 2015, pp. 1–17.
Vergoossen, L. W. *et al.* (2019) 'White Matter Connectivity Abnormalities in Prediabetes and Type 2 Diabetes: The Maastricht Study', *Diabetes Care*. American Diabetes Association, p. dc190762.

Wallace, T. M., Levy, J. C. and Matthews, D. R. (2004) 'Use and abuse of HOMA modeling.', *Diabetes care*. American Diabetes Association, 27(6), pp. 1487–95.

Weinstein, G. *et al.* (2015) 'Glucose indices are associated with cognitive and structural brain measures in young adults', *Neurology*, 84(23), pp. 2329–2337.

Xiong, Y. *et al.* (2016) 'A diffusion tensor imaging study on white matter abnormalities in patients with type 2 diabetes using tract-based spatial statistics', *American Journal of Neuroradiology*. American Society of Neuroradiology, 37(8), pp. 1462–1469.

Xue-shan, Z. *et al.* (2016) 'Imbalanced cholesterol metabolism in Alzheimer's disease', *Clinica Chimica Acta*, 456, pp. 107–114.

Yarchoan, M. and Arnold, S. E. (2014) 'Repurposing diabetes drugs for brain insulin resistance in Alzheimer disease.', *Diabetes*. American Diabetes Association, 63(7), pp. 2253–61.

Zetterberg, H., Rohrer, J. D. and Schott, J. M. (2018) 'Cerebrospinal fluid in the dementias', *Handbook of Clinical Neurology*. Elsevier, 146, pp. 85–97.

Zhuo, Y. *et al.* (2019) 'White matter impairment in type 2 diabetes mellitus with and without microvascular disease', *NeuroImage: Clinical*. Elsevier Inc., 24.