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Abstract

Alzheimer's disease (AD) is the most common form of progressive neurodegenerative disorders afflicting more than 47 million people worldwide. This poses a significant economic cost and highlights the need to develop disease-modifying therapeutic strategies. AD is characterised by the formation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles. It has been shown that the 37kDa/67kDa laminin receptor (LRP/LR) and telomerase are implicated in the pathogenesis of AD through their respective interactions with Aβ and the AD-associated proteins: the amyloid precursor protein (APP), β- and γ-secretase. Aβ inhibits telomerase activity and LRP/LR has a function in regulating telomerase activity as well as in the internalisation and shedding of Aβ. We have previously shown *in vitro*, that blockade of the 37kDa/67kDa Laminin Receptor (LRP/LR) with the anti-LRP/LR specific antibody, IgG1-iS18, resulted in reduced Aβ-induced cytotoxicity and Aβ accumulation. The current study consisted of two parts, *in vivo* analysis to determine the effect of blocking LRP/LR with IgG1-iS18 and *in vitro* analysis on the overexpression of LRP intracellularly. To test the effect of blocking LRP/LR on Aβ formation and AD associated symptoms, 5XFAD AD transgenic mice received IgG1-iS18 through intranasal administration. The 5XFAD mice harbour three mutant human APP (695) and two PS1 Familial AD mutations to induce amyloid pathology through overexpression of APP. Furthermore, the resultant effects on the expression of the AD-related proteins, mTERT and γH2AX expression were investigated. We show that this treatment resulted in an improvement in memory, decreased vacuolization and Aβ plaque formation. Moreover, a significant decrease in $A\beta_{42}$ protein expression with a concomitant increase in APP and telomerase reverse transcriptase (mTERT) levels was observed. These data indicate IgG1-iS18 as a potentially powerful therapeutic tool for AD. To investigate the effect of overexpressing LRP in *in vitro* AD models, the SH-SY5Y neuronal and HEK293 AD cell culture models were stably transfected with pCIneo-moLRP::FLAG, to induce overexpression of LRP. This treatment resulted in increased LRP and hTERT levels, with co-localisation between the LRP and hTERT, together with a concomitant decrease in Aβ levels. Additionally, this treatment increased telomerase activity and rescued cells from Aβ-mediated cytotoxicity. These data suggest that overexpression of LRP intracellularly might represent a potential therapeutic strategy for treatment of AD. Taken altogether, targeting LRP/LR in an AD context has provided insight into the disease and simultaneously provided alternative therapeutic options for halting AD progression.

1. Introduction

1.1. Alzheimer's disease epidemiology

Alzheimer's disease (AD) is the most common form of progressive neurodegenerative disorders afflicting in excess of 47 million people on a worldwide basis, of which there are approximately 186, 000 people living with Dementia in South Africa. These numbers are expected to double by 2030 and more than triple by 2050 (Alzheimer's Disease International, 2015). Dementia causes overwhelming effects on those suffering from it, as well as their caregivers, families, and societies physically, psychologically and economically. Unfortunately, lack of awareness and understanding of Dementia in most countries results in stigmatization as well as barriers to both diagnosis and care. This most prevalent form of Dementia predominantly affects the ageing population and is of significant economic cost, whereby in 2015, the global cost was estimated at 818 billion USD (Alzheimer's Disease International, 2015). In 2015, 9.9 million new cases of Dementia were reported (Alzheimer's Disease International, 2015), which equates to one every three seconds. The World Health Organization has ranked South Africa 31st in the world in terms of death rate for AD/Dementia and this statistic is based on underrepresented data, attributable to a lack of epidemiological data to provide representative estimates. Additionally, due to the knowledge gap which currently exists in the understanding of the disease-causing mechanism and that presently, only palliative therapeutic options are available, research based on the development of therapeutic strategies for AD is of high priority.

It is of great concern that South Africa's public healthcare system is principally focussed on addressing primary healthcare needs such as HIV/AIDS and tuberculosis. In reality, more than 19.4 million people in eastern and southern Africa are living with HIV/AIDS, with an estimated 790 000 people dying from the devastating disease in these regions, in 2016 (UNAIDS Fact Sheet, 2017). Many of the deceased are young parents, and this leaves the burden of childcare on the aged and so often the elderly grandparents suffering with Dementia are left without children to care for them. The reality is that government spending will continue to address the high demands associated with primary healthcare needs. Whilst there is an understanding of this need, this only highlights the alarming statistics which provide proof of the necessity for disease-modifying treatment strategies for neurodegenerative disorders such as AD. Ultimately, there is a great need for readily

available and inexpensive treatments which impede the progression of AD, thus reducing the need for care and thus reducing costs on all fronts.

1.2. Alzheimer's disease pathology and molecular mechanisms

AD is characterised by two key neuropathological hallmarks: (i) the accumulation of the neurotoxic amyloid beta-42 ($A\beta_{42}$) peptide, resulting in the development of extracellular amyloid beta plaques (Xiao *et al*., 2015), thereby causing neuronal loss (Serrano-Pozo *et al*., 2011) and (ii) the aggregation of hyper-phosphorylated tau (a microtubule associated protein), producing intracellular neurofibrillary tangles, leading to synaptic loss (Avila, 2006). Collectively, it is these factors and their associations, which cause the progressive and devastating behavioural and cognitive dysfunction symptoms as seen in those suffering from AD.

The 4-kDa Aβ peptide is the candidate etiological cause for AD, expressly, it is the 42 amino acid (aa) Aβ peptide which amasses to cause the neurotoxic effects present in AD (Choi *et al*., 2014). Generation of the Aβ peptide occurs via the amyloidogenic pathway, when the amyloid precursor protein (APP) in the cell membrane, is sequentially cleaved by β-secretase (also known as BACE1 – B-site APP cleaving enzyme) and γ-secretase (the catalytic subunit is referred to as PS1) (Fig.1). Initially, APP is cleaved at the N-terminus by β-secretase, thus releasing the secreted APPβ (sAPPβ) fragment. Thereafter, Aβ shedding occurs, whereby γ-secretase cleaves the remaining C-terminus of APP to release the small Aβ fragment into the extracellular environment (Caetano *et al*., 2011) (Fig.1). Under standard physiological conditions, APP is processed through the non-amyloidogenic pathway. In this context, APP is cleaved, initially by α-secretase to produce sAPPα (which precludes Aβ formation), thereafter, by γ-secretase to finally produce p3. Although the physiological functions of these products are not well understood, it is suggested that sAPPα has growth promoting functions, whereby it plays a role in the development of the brain. In addition, sAPPα may enhance neurite growth, improve the formation of memories and contribute to pro-survival pathways (review see Chow *et al*., 2010). AD occurs when the amyloidogenic pathway is inappropriately favoured and when $A\beta_{42}$ degradation is reduced, thus leading to accumulation of these peptides (Kakiya *et al*., 2012). It is proposed that the mechanism by which Aβ prompts the characteristic neuronal loss is due to its interaction with specific cell components (Verdier & Penke, 2004; Da Costa Dias *et al*., 2011). This occurs when the Aβ either directly interacts with receptors on the cell surface (Da Costa Dias *et al*., 2011) or through indirect

interactions, such as incorporation into cell organelles and lipid membranes (Verdier & Penke, 2004). These processes modify signal transduction pathways, such as disruptions in cholinergic systems (Kelly *et al*., 1996) consequently causing synaptic dysfunction and neuronal death. The core component of amyloid plaques is the $A\beta_{42}$ peptide, it has a higher propensity to aggregate (Jarrett *et al*., 1993) and is known to be more neurotoxic (Saido, 1998) than its 40 amino acid ($A\beta_{40}$) counterpart, which is predominant in non-diseased brains. The plaques themselves are thought to not directly cause the AD pathology but rather, the pathology occurs as a consequence of Aβ-mediated distortions in the neural morphology. These distortions impede neurotransmission and correspondingly, cause neural dysfunction (Hyman *et al*., 1995). Instead, it is the soluble Aβ oligomers which are accepted as the pathological agent due to their neurotoxicity, caused by their functional role in forming ion-permissible channels in the cell membrane. Ultimately, resulting in an influx of ions, specifically Ca^{2+}, with subsequent cytotoxicity (Lin *et al*., 2001; Demuro *et al*., 2005). Aβ neurotoxicity has therefore been linked to Aβ-induced neuronal apoptosis through this disruption in cellular calcium homeostasis as well as oxidative stress, both of which contribute to mitochondrial dysfunction (Mattson, 1997) and DNA damage (Zhang *et al*, 1995; Deng *et al*, 1999).

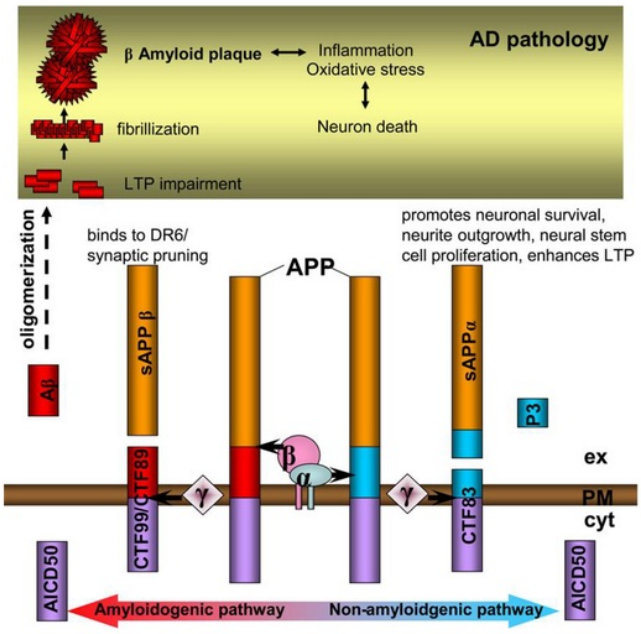

Fig. 1: Schematic of the processing of APP and the cleavage products. Pathway of non-amyloidogenic processing of APP (right/blue) involves cleavage by α- and γ-secretases generating the secreted form of APP (sAPPα) and C-terminal fragments (CTF 83, p3 and AICD50). Pathway of amyloidogenic APP processing (left/red) involves cleavage by β- and γ-secretases generating the secreted form of APP (sAPPβ), C-terminal fragments (CTF 99 and CTF 89) and Aβ. (Adopted directly from Chow *et al*., 2010)

However, additional aspects, which contribute to AD include: lipid oxidation, oxidative stress and protein degradation, all of which are attributed to the abovementioned interaction between Aβ and cell surface receptors (Bartzokis, 2011; Marchesi, 2011; Da Costa Dias *et al*., 2011). This interaction results in the internalisation of $A\beta_{42}$, thus causing the intracellular accumulation of $A\beta_{42}$, which directly corresponds with the progression of AD (Stefani, 2010). Once $A\beta_{42}$ is internalised, aggregation of the $A\beta_{42}$ oligomers occurs, subsequently resulting in the aforementioned disturbance in cell signalling, dysfunction of normal cellular processes and cellular damage, eventually causing cell death (Mucke & Selkoe, 2012).

One of the receptors in AD which interacts with Aβ, is the 37kDa Laminin Receptor Precursor/67kDa high affinity Laminin Receptor (LRP/LR) (Da Costa Dias *et al*., 2013).

1.3. The 37 kDa Laminin Receptor Precursor/ 67 kDa high affinity Laminin Receptor (LRP/LR) and its role in Alzheimer's disease.

LRP/LR, otherwise known as LamR1, RPSA and p40 is a multifunctional type II transmembrane receptor consisting of 295aa and is predominantly found within lipid raft regions of the plasma membrane, however, it also exists within the cytoplasm and the nucleus (Mbazima *et al*., 2010). It was suggested that the 37 kDa LRP gives rise to the 67 kDa LR, however, it is now understood that the 67 kDa LR is not a homodimer of the 37 kDa LRP (Hundt *et al*., 2001) but rather, it is a result of post-translational modifications (Landowski *et al*., 1995). LRP/LR has a substantial physiological role in both normal and cancerous cells. This is due to the interactions of LRP/LR as a receptor in the extracellular environment, whereby it binds to elastin, laminin-1, heparin sulfate proteoglycans (HSPGs), prion proteins- (PrP^{c} and PrP^{Sc}) (Gauczynski *et al*., 2006) and Aβ (Jovanovic et al., 2015). In the intracellular environment, LRP/LR interacts with histones (Kinoshita *et al*., 1998) to maintain nuclear structures, it acts as a component of the 40S ribosomal subunit, thereby mediating translation (Ford *et al*., 1999) and it interacts with the cytoskeletal proteins, tubulin and actin (Venticinque *et al*, 2011). This further indicates an integral role in translation and cell migration (for review on LRP/LR see Jovanovic *et al*., 2015; Weiss, 2017). However, it is of particular importance, that these interactions associate LRP/LR with numerous diseases. These include, but are not limited to, cancer, prion disorders, ageing and AD. In cancer, LRP/LR is known to be highly upregulated, leading to enhanced adhesion and invasion and ultimately, metastasis, as well as angiogenesis and the inhibition of apoptosis (Rao *et al*., 1989; Zuber *et al*., 2008; Omar *et al*., 2012; Moodley & Weiss., 2013; Khumalo *et al*., 2013;

Khusal *et al*., 2013; Chetty *et al*., 2014; Rebelo *et al*., 2016; Vania *et al*., 2016). LRP/LR is involved in the ageing process due to an interaction with telomerase (Naidoo *et al*., 2015; Otgaar *et al*., 2017). In AD, LRP/LR is implicated through its interaction with Aβ as well as the AD-related proteins; APP, γ- secretase and β- secretase (Jovanovic *et al*., 2014).

It has been demonstrated that LRP/LR and Aβ co-localise on the cell surface and that LRP/LR is a receptor for synthetic $A\beta_{42}$ peptides (Da Costa Dias *et al*., 2013). When antibody (IgG1-iS18) and small hairpin RNA (shRNA) technologies were employed to target LRP/LR, to block the receptor and downregulate its expression, respectively, it was observed that both treatments significantly reduced Aβ mediated cytotoxicity and Aβ shedding (Jovanovic *et al*., 2013; Da Costa Dias *et al*., 2013). With this information, it was discovered that LRP/LR is implicated in the pathogenicity of AD and this warranted further study. It was observed that the interaction between $A\beta_{42}$ and LRP/LR on the cell surface inhibits cell proliferation and induces apoptosis, as indicated by the presence of apoptotic bodies post-treatment with cytotoxic levels of $A\beta_{42}$ peptides (Da Costa Dias *et al*., 2014). This is due to the involvement of LRP/LR in the internalisation of $A\beta_{42}$, thus resulting in the accumulation of $A\beta_{42}$ intracellularly (Jovanovic *et al*., 2014) and ultimately $A\beta_{42}$ mediated cytotoxicity (Da Costa Dias *et al*., 2013). Furthermore, as PrP^c binds to both Aβ and LRP/LR, it was investigated whether the underlying mechanism of the $A\beta_{42}$ – induced neuronal cytotoxicity was an indirect result of the PrP^c - $A\beta_{42}$ interaction (Pinnock et al., 2016). It was observed *in vitro* that overexpression of PrP^c caused a significant enhancement in $A\beta_{42}$ mediated cytotoxicity. However, on blockade of LRP/LR with IgG1-iS18, cell viability was significantly increased in PrP^c expressing cells but not in PrP^c negative cells. Therefore, suggesting LRP/LR has a significant function in PrP^c -mediated, $A\beta_{42}$ – induced cytotoxicity (Pinnock et al., 2016). Therefore, LRP/LR is pivotal in causing the resultant aforementioned harmful cellular effects through the facilitation of the uptake of $A\beta_{42}$ (Jovanovic *et al*., 2014). In addition, Jovanovic *et al*, 2014 observed that LRP/LR co-localises with APP, β- and γ – secretases inside the cell and on the cell surface. Interestingly, both the IgG1-iS18 and shRNA treatments do not alter the expression of APP, β- and γ –secretases on the cell surface but rather reduce levels of sAPPβ, and the release of Aβ into the extracellular environment (Jovanovic *et al*., 2013; Da Costa Dias *et al*., 2013). Upon further investigation, the interaction between LRP/LR and β – secretase was found to be indirect. However, the interaction with PS1, the catalytic subunit of γ-secretase and LRP/LR was established as direct and that this interaction enhances cleavage of APP, thus releasing sAPPβ. Therefore,

the blockade or knockdown of LRP/LR results in the impediment of β- and γ-secretase activity, through the impediment of the interaction between the two, respectively, thereby reducing sAPPβ levels and ultimately decreasing Aβ shedding (Jovanovic *et al*., 2013) (Fig.2).

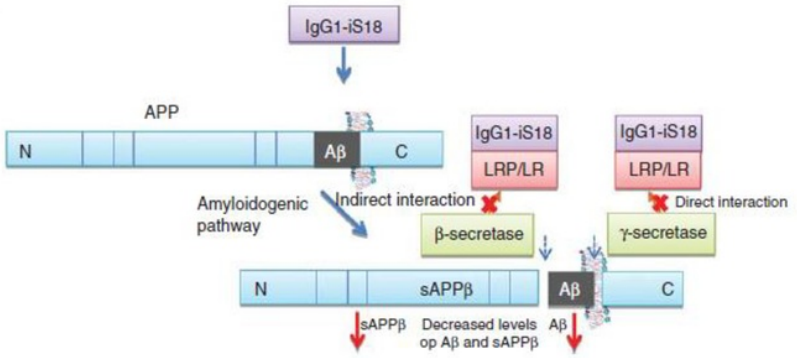

Fig. 2. The effects of anti-LRP/LR specific antibody IgG1-iS18 on the amyloidogenic processing of APP. LRP/LR blockade with IgG1-iS18 reduces the amount of sAPPβ and Aβ generated. (Adopted directly from Jovanovic *et al*., 2015).

Taken altogether, there is a definitive role, which LRP/LR plays in AD. In addition, strategies employed to target LRP/LR, inclusive of blockade by the anti-LRP/LR specific antibody, IgG1-iS18 and downregulation via anti-LRP shRNA, significantly reduce Aβ shedding as well as internalisation. This information is therefore, invaluable in the search and development of therapeutics for the treatment of AD.

1.4. The role of telomerase and telomere biology in Alzheimer's disease

1.4.1. Telomerase in the maintenance of telomeric structures

Recently, an additional protein found to be associated with AD is telomerase, a ribonucleoprotein with reverse transcriptase activity, which is principally found in highly proliferative cells (Greider & Blackburn, 1987; Kim *et al*., 1994). Telomerase is a multi-subunit protein but has two key components (Fig. 3). The catalytic subunit, the telomerase reverse transcriptase, TERT and the telomerase RNA component (TERC), serving as the RNA template, utilized by TERT to extend telomeres (Nakamura and Cech, 1998). Telomerase has a key function in protecting DNA from degradation, through the addition of telomeric (TTAGGG) repeats to the ends of telomeric DNA in a 3′- 5′ direction (Fig. 3). Therefore, the principal function of telomerase is to maintain and elongate telomeres (Greider

& Blackburn, 1987; Kim *et al*., 1994).This function of telomerase is essential due to the "end replication problem", which occurs as a result of the semi-conservative mechanism of DNA polymerase (Lingner & Cech, 1998).TERT serves as the limiting factor of telomerase activity, as the activity is directly dependent on the amount of TERT present (Bodnar *et al*., 1998; Counter *et al*., 1998).

Telomerase activity has a key role in cellular senescence and immortalisation, it therefore, plays a significant role in the ageing process and the cancerous state (Shay & Wright, 2005). Cellular senescence occurs as a result of cellular stresses, which include telomere erosion, genomic instability and mitochondrial dysfunction. All of which lead to functional decline and disruptions in tissue homeostasis due to a limit in the replicative and regenerative potential of cells (Allsop & Harley, 1995; Molofsky *et al*., 2006). Furthermore, in the context of cancer, senescence as a result of these stresses is bypassed due to an increase in telomerase activity (Shay & Wright, 2005).

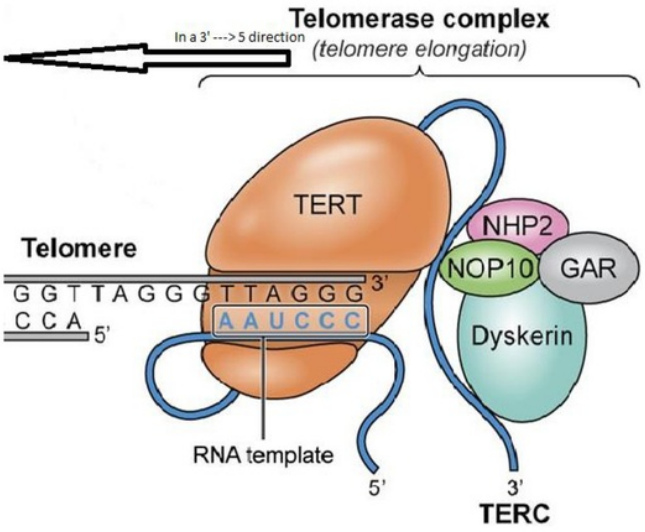

Figure 3. Schematic diagram of the multi-subunit protein, telomerase, elongating telomeric DNA. Telomere elongation occurs when the two essential subunits, TERT and TERC catalyse the addition of TTAGGG repeats to the ends of chromosomes. These are indicated in a complex together with the telomerase related proteins, Dyskerin, NHP2, NOP10 and GAR which are partly responsible for the stability of the complex, as well as for the extra-telomeric functions of telomerase. (Adopted directly from Townsley *et al*., 2014).

1.4.2. Telomerase and its non-telomeric functions: Protection

There are various extra-telomeric processes in which telomerase plays a part, predominantly for the conservation of cell viability (Saretzki, 2014). In addition to its role as the catalytic subunit of telomerase, TERT localizes to the mitochondria, whereby it aids in the functioning of the organelle, as well as serving a protective purpose (Ahmed *et al*., 2008; Haendler *et al*., 2009; Singhapol *et al*., 2013). This is evident when reactive oxygen species (ROS) are present and conditions become hypoxic, TERT translocates to the mitochondria. Here, TERT protects the organelle against mitochondrial DNA (mtDNA) damage, as well as apoptosis (Cong & Shay, 2008). Additionally, TERT contributes towards the replication and repair of mtDNA, altogether demonstrating TERT is essential to the function of the mitochondria (Sharma *et al*., 2011).

Furthermore, telomerase has been implicated in DNA repair and DNA damage responses by which it is involved in the recruitment of DNA repair proteins (Saretzki, 2014). Moreover, there is further evidence to suggest the role of telomerase in DNA repair by the occurrence of telomeric repeats at DNA breaks (Cong & Shay, 2008). There are several factors involved in the DNA repair process as well as signalling the presence of DNA damage, which accumulate at the occurrence of double-stranded DNA breaks. One of particular importance is the phosphorylation of histone H2AX (γH2AX) (Paull *et al*, 2000), which promotes the recruitment of repair and damage proteins. It has been elucidated that a lack of hTERT in cells, impairs the DNA damage response due to a reduction in the amount of H2AX phosphorylation (Masutomi *et al*, 2005). Therefore, this suggests that hTERT has an essential role in regulation of the DNA damage response pathway.

1.4.3. Telomerase and its non-telomeric functions: Transcriptional regulation

Additionally, TERT is known to play a role as a transcriptional regulator, whereby it serves as a transcriptional activator of numerous genes related to signal transduction pathways involved in cell viability as well as development and proliferative processes that are regulated by the Myc and Wnt pathways (Choi *et al.,* 2008; Cong & Shay, 2008; Park *et al*., 2009). Thus, indicating a complex set of regulatory processes which occur amongst the aforementioned pathways and TERT itself (Choi *et al.,* 2008; Park *et al*., 2009; Saretzki, 2014). TERT expression is known to be activated by the transcription factor, Myc and there is evidence proposing that a positive feedback loop exists between the two (Choi *et al.,* 2008; Park *et al*., 2009). In addition, TERT plays a regulatory role through an association with the

Wnt pathway, whereby both are involved in controlling entry into the cell cycle and therefore, proliferation (Cong & Shay, 2008). This occurs as TERT occupies regions on the promoters of the genes of the cell cycle transcriptions factors, Myc and cyclin D1 (Yang *et al*., 2008; Li *et al*., 2011). An alternative mechanism for activation of the Wnt signalling pathway is by the transcription factor β-catenin, of which TERT has been shown to act as a co-factor, thereby further indicating an involvement in the promotion of cell viability and proliferation (Park *et al*., 2009). Therefore, there is a clear indication that TERT is crucial in the regulation of cell survival and proliferative pathways, in addition to its role as the catalytic subunit of telomerase.

1.4.4. The role of telomerase in Alzheimer's disease

There is fairly recent evidence that has implicated telomerase in the pathological processes of AD (Zhu & Mattson., 2000; Franco *et al*., 2006; Wang *et al*., 2015). There is supporting evidence which has shown that AD sufferers present shorter telomere lengths in their neuronal and T cells (Franco *et al*., 2006). Furthermore, it has been demonstrated *in vitro*, that telomerase activity is inhibited by $A\beta_{42}$ and that this occurs when the Aβ oligomers physically bind to the telomeric DNA-RNA template complex of telomerase (Wang *et al*., 2015), which is indicative of an antagonistic relationship that exists between telomerase and Aβ within neurons. Thus, this process actively contributes to the telomere loss experienced by AD patients. It has been proposed that an upregulation of telomerase could be a potential therapeutic strategy for the treatment of AD as the resulting overexpression of TERT plays a protective function against Aβ-induced apoptosis in neuronal cells (Zhu & Mattson., 2000). Furthermore, it has been shown that TERT interacts with the tumour suppressor, p53, through an interaction with the TERT binding protein, TEP-1 (Li et al., 1999a). p53 is known to induce apoptosis through ROS-induced alterations in mitochondrial membrane potential and is present at increased levels in amyloid plaque affected neurons (de la Monte et al., 1997), as well as in mouse brain tissue overexpressing Aβ (LaFerla et al., 1996). Therefore, TERT is involved in modulating apoptotic effects of p53. A novel discovery by Naidoo *et al*., 2015 has illustrated that there is both an interaction and co-localisation between LRP/LR and hTERT within perinuclear compartments and on the cell surface. In addition, it was shown that small interfering RNAs (siRNAs) directed against LRP/LR, facilitating its knockdown, resulted in a significant reduction in telomerase activity (Naidoo *et al*., 2015). Furthermore, Otgaar *et al*., 2017 elucidated that overexpression of LRP::FLAG (Vana & Weiss, 2006) resulted in an increase in TERT expression, telomerase activity, telomere length as well as a

reduction in senescent markers (Otgaar *et al*., 2017). Altogether indicating that LRP/LR has a role in the regulation of telomerase activity and TERT expression (Naidoo *et al*., 2015; Otgaar *et al*., 2017). Therefore, employing strategies, such as the overexpression of LRP::FLAG, which target LRP/LR, to increase TERT levels and telomerase activity could provide a prospective therapeutic strategy for the treatment of AD.

1.5. Hypothesis

There is a clear demand for non-palliative therapeutic strategies for AD, this is highlighted by the high occurrence of AD, the significant economic cost thereof and due to the current availability of treatments being merely palliative in nature. LRP/LR plays an important role in facilitating the pathological processes involved in AD. LRP/LR functions as a receptor for neurotoxic Aβ peptides and is involved in the amyloidogenic processing of APP, due to its interaction with β-and γ-secretase. Furthermore, LRP/LR plays a critical role in the internalisation of Aβ and therefore, its accumulation and ultimately the resulting cytotoxic effects initiated by Aβ and the related pathways. In addition, telomerase is known to contribute to the pathological processes in AD. This is evident in the shortened telomeres of AD sufferers, as well as the antagonistic relationship between $A\beta_{42}$, the aetiological agent of AD, and TERT, the reverse transcriptase of telomerase. Moreover, it has been elucidated that LRP/LR has a regulatory role in telomerase activity and TERT expression. Furthermore, this interaction between LRP/LR and telomerase had been tested in an ageing and a cancer context but had yet to be assessed in the context of AD.

As aforementioned, studies performed on the knockdown and blockade of LRP/LR through shRNA and LRP specific antibodies in an *in vitro* AD model have shown that impediment/inhibition of LRP/LR has positive effects on AD pathology. This includes rescue from Aβ-mediated cytotoxicity and a significant reduction of Aβ internalisation and shedding, thereby suggesting the blockade/knockdown of LRP/LR as a potential therapeutic strategy for AD. However, since these studies were performed *in vitro*, it was of importance to continue this study in an *in vivo* model to further validate this strategy. Therefore, the first part of this study included treating AD transgenic mice with IgG1-iS18 and thereafter assessing the resulting effects on AD pathology, including the effects on cognitive function, AD histopathology and the levels of the AD-related proteins.

It has been suggested that upregulating TERT could be a potential therapeutic strategy for AD, due to the resultant protection from Aβ-induced apoptosis. Therefore, the second part of

this study served to assess the effects of overexpressing LRP::FLAG intracellularly, with a resultant increase in TERT levels, on cell viability, telomerase activity and $A\beta_{42}$ levels in *in vitro* AD models.

4. Results

4.1. Alzheimer's disease transgenic mouse study

To validate whether targeting LRP/LR using IgG1-iS18, in 5XFAD transgenic mice, would influence their cognitive abilities, the novel object recognition and puzzle box tests were performed by Dr. E. Ferreira- van der Merwe. The novel object recognition test was used to evaluate the effect of treatment with IgG1-iS18 on recognition memory in comparison to PBS treated control mice. Dr. Ferreira-van der Merwe observed that the treated mice exhibited object recognition by preferentially exploring the novel object (58.09%) compared to the familiar object (41.91%) during the testing phase (Fig. S1A). To further investigate the short and long-term memory as well as learning ability of the AD transgenic mice, Dr. Ferreira-van der Merwe performed the puzzle box test. Here, the data suggested that IgG1-iS18 significantly improved short term memory as well as learning ability (Fig. S1B). Therefore, overall, it was observed there was a significant improvement in short term and learning memory after intranasal treatment with IgG1-iS18 (Ferreira, Bignoux, Otgaar, *et al.* Oncotarget, *in press*).

To further validate IgG1-iS18 as a potential therapy for the treatment of AD, 16 AD transgenic mice (8 treated with IgG1-iS18 and 8 treated with PBS) were sacrificed at 9 months of age to perform immunohistochemical studies and biochemical testing. Refer to Fig. 4 for an overview of the design of the *in vivo* study. These tests and all further data analysis were performed by both Dr. Ferreira-van der Merwe and myself.

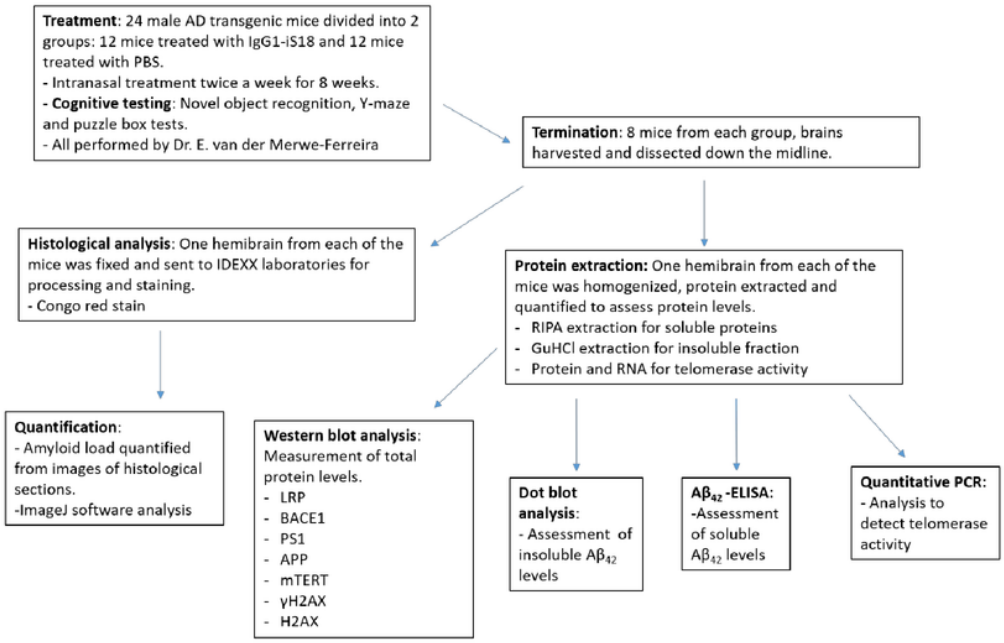

Fig. 4: Design of the Alzheimer's disease *in vivo* study. A brief outline of the experimental procedures performed to assess the efficacy of IgG1-iS18 as a therapeutic for AD in 5XFAD AD transgenic mouse models.

4.1.1. Treatment with IgG1-iS18 decreases AD histopathological hallmarks

Considering the improvement in memory after treatment with IgG1-iS18, we decided to investigate the effect on the histopathology of the hippocampus of the AD mice. Histopathological analysis of the hippocampus region of the brains of the IgG1-iS18 and PBS treated mice was performed using the Congo red stain. Images were taken at 400 X magnification, Aβ plaques are stained red and amyloid load was quantified using ImageJ software.

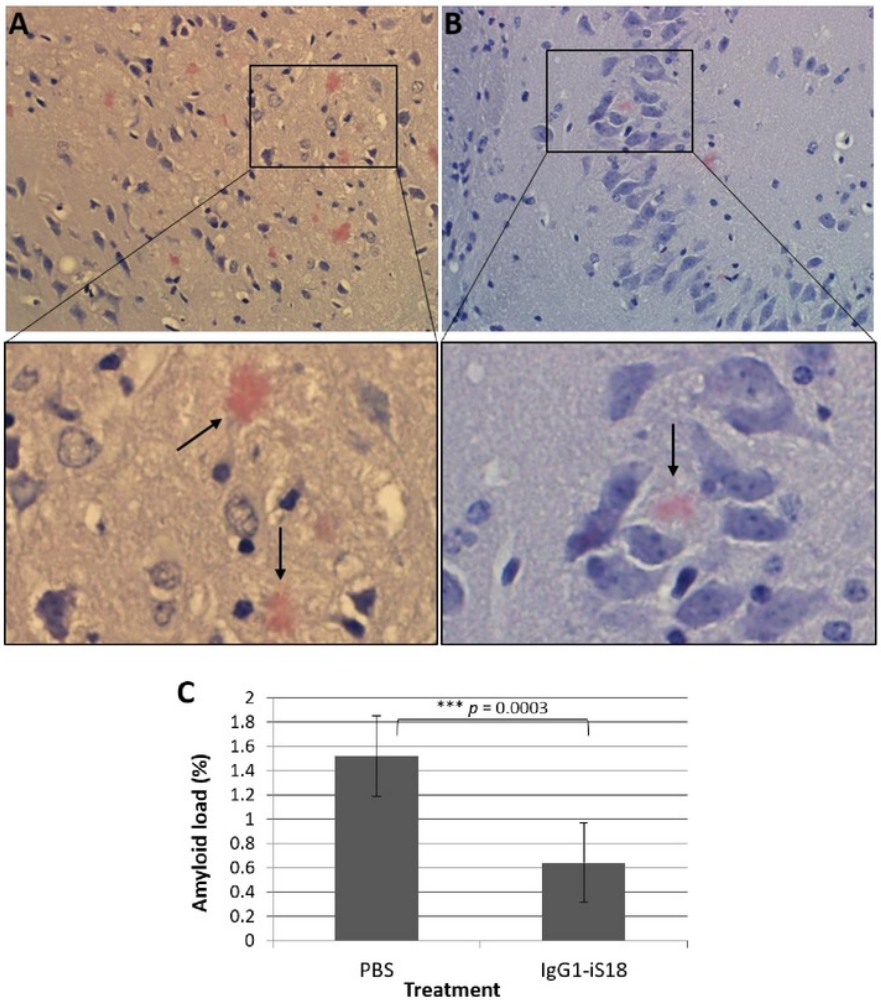

Fig. 5: Congo red stain of the hippocampus of AD transgenic mice treated with A) PBS and B) IgG1-iS18. Magnification at 400 X. C) Amyloid load (%) is expressed as the proportion (%) of tissue area occupied by amyloid beta plaques in the hippocampal sections of the PBS and IgG1-iS18 treated mice. A decrease in amyloid plaque and vacuole formation is observed after treatment with IgG1-iS18. Error bars represent standard deviation, n=7. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (Adapted from Ferreira, Bignoux, Otgaar, *et al*. Oncotarget, *in press*).

Quantitative and post hoc analysis of amyloid load (%) in hippocampal regions of the mouse brains was performed. It was determined that the IgG1-iS18 treated mice [Fig. 5B (Mean = 0.64%, SD = 0.32%)] presented a significantly lower [F (1, 12) = 25.08, p = 0.00031] amyloid load (Fig. 5C) when compared to the hippocampal regions of PBS treated mice [Fig.5A (Mean = 1.52%, SD = 0.33%)].

4.1.2. Intranasal administration of IgG1-iS18 decreases soluble and insoluble Aβ levels in brains of AD transgenic mice

Since a significant decrease in amyloid plaque formation was seen upon histopathological analysis, we thereby wanted to confirm the effect of the IgG1-iS18 treatment on the Aβ protein levels. Therefore, we quantified $A\beta_{42}$ protein levels in the contralateral hemispheres of both treatment groups by performing dot-blot analysis of GuHCl-soluble (insoluble) (Fig.6A) and an $A\beta_{1\text{-}42}$-ELISA on the Tris-soluble (soluble) (Fig. 6B) samples.

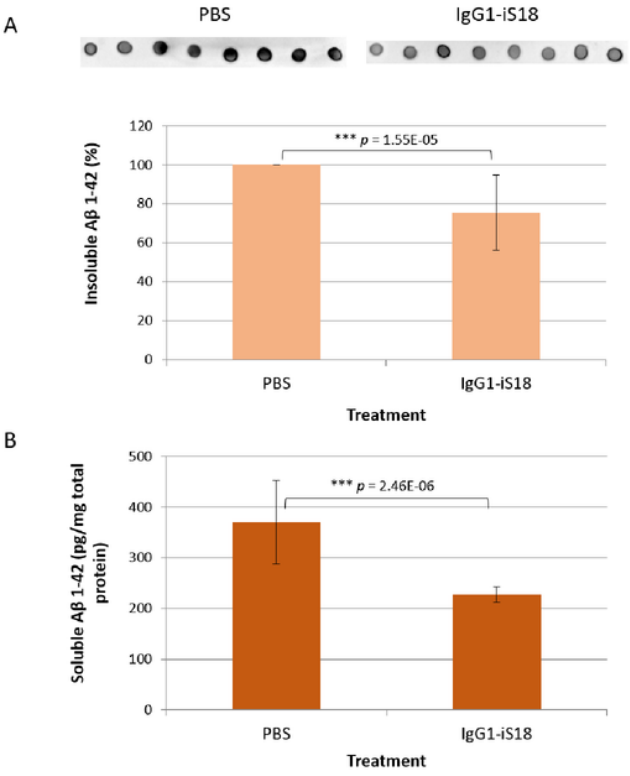

Fig. 6: Levels of Aβ in the brain tissue of AD transgenic mice after treatment with IgG1-iS18 and PBS. (A) Insoluble Aβ levels were determined by dot blot and calculated as a percentage per mg brain tissue. Data shown (average ± standard deviation) is representative of eight biological repeats (performed in duplicate) per treatment group. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (B) Levels of soluble $A\beta_{42}$ in brain tissue of AD transgenic mice after treatment with IgG1-iS18 and PBS as determined by $A\beta_{42}$ ELISA. Data shown (average ± standard deviation) was calculated as pg $A\beta_{42}$ per mg total protein and is representative of seven biological repeats (performed in duplicate) per treatment group. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (Adapted from Ferreira, Bignoux, Otgaar *et al*. Oncotarget, i*n press*).

Upon dot-blot analysis, it was shown that IgG1-iS18 treated mice exhibited a significant 24% decrease (p=1.55E-05) in insoluble $A\beta_{42}$ levels per mg of brain tissue when compared to the PBS control (Fig.6A). In addition, ELISA analysis of the soluble $A\beta_{42}$ levels revealed that the antibody treated mice hemi-brain homogenates contained an average of 227.71 pg $A\beta_{42}$ /mg total protein, whereas the PBS control hemi-brain homogenates contained an average 370.21 pg $A\beta_{42}$ /mg total protein. This presented a significant decrease of 38.49 % (p=2.46E-06) in soluble $A\beta_{42}$ levels in the brains of the mice treated with IgG1-iS18 (Fig.6B). Altogether, this data coincides with the data presented upon histopathological analysis (Fig. 5), whereby the antibody treatment showed a significant reduction in amyloid plaque formation.

4.1.3. Treatment with IgG1-iS18 significantly increases APP levels

Since it was formerly reported that LRP, β-secretase, γ-secretase and APP levels were not affected upon treatment with IgG1-iS18 *in vitro* (Jovanovic *et al.*, 2013), we therefore wanted to confirm whether the levels of these proteins had indeed remained unchanged in the brains of both treatment groups. Thus, we examined these levels via western blot with subsequent densitometric analysis.

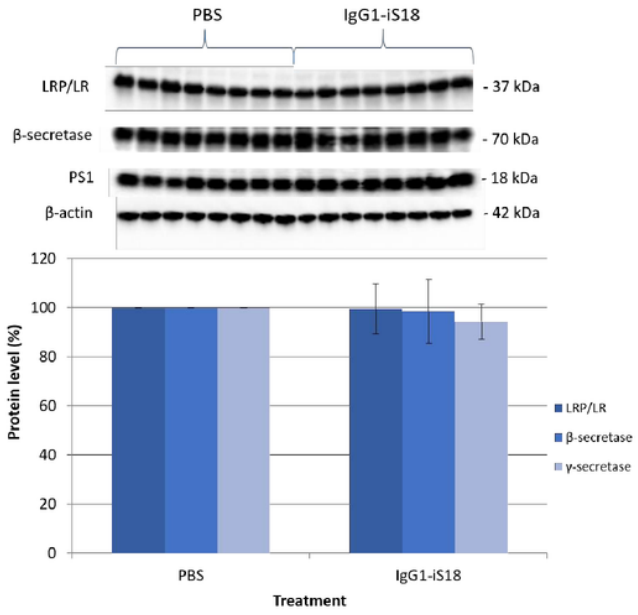

Fig. 7: Western blot analysis of LRP/LR, β- and γ-secretase protein levels in brain tissue of AD transgenic mice after treatment with IgG1-iS18 and PBS. No significant difference in LRP/LR, β- and γ-secretase protein levels were observed. Error bars represent standard deviation, n= 8. (Adapted from Ferreira, Bignoux, Otgaar *et al.* Oncotarget, *in press*).

Since IgG1-iS18 merely blocks the interaction between LRP and the β -, γ-secretases respectively, we expected that this would not affect the respective protein expression levels (Jovanovic *et al.*, 2014). As anticipated, we found no significant difference in the levels of LRP, β-secretase and γ-secretase when comparing the levels of the respective proteins in the

hemi-brain homogenates of the antibody treated and PBS treated mice (Fig. 7). However, in contrast to what was previously observed *in vitro*, we found APP levels to be significantly higher on average (85.86%; p=4.94E-06) in the hemi-brain homogenates of the IgG1-iS18 treated mice when compared to the APP levels in the brains of the PBS control mice (Fig. 8). Therefore, we suggest this finding in the *in vivo* study to be attributed to a reduction in cleavage of this protein by β- and γ-secretase as due to the reduced interaction between LRP and the secretases, as there is a concomitant reduction in $A\beta_{42}$ levels (Fig. 6)

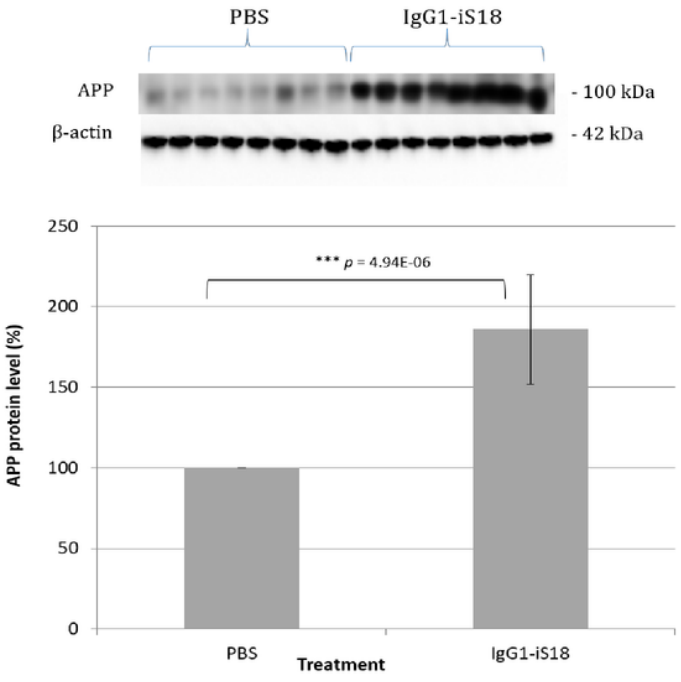

Fig. 8: Western blot analysis of APP levels in brain tissue of AD transgenic mice after treatment with IgG1-iS18 and PBS. A significant increase in APP levels was observed after treatment with IgG1-iS18. Error bars represent standard deviation, n=8. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (Adapted from Ferreira, Bignoux, Otgaar *et al*. Oncotarget, *in press*).

4.1.4. IgG1-iS18 treatment substantially increases mTERT expression and phosphorylation of H2AX

As aforementioned, LRP/LR co-localises with the telomerase reverse transcriptase, TERT (Naidoo *et al*., 2015) and TERT has been reported to play a neuroprotective role (Zhu *et al*.,

2001). Thus, we resolved to assess whether treatment with the IgG1-iS18 antibody affected the levels of mTERT in the mouse brain.

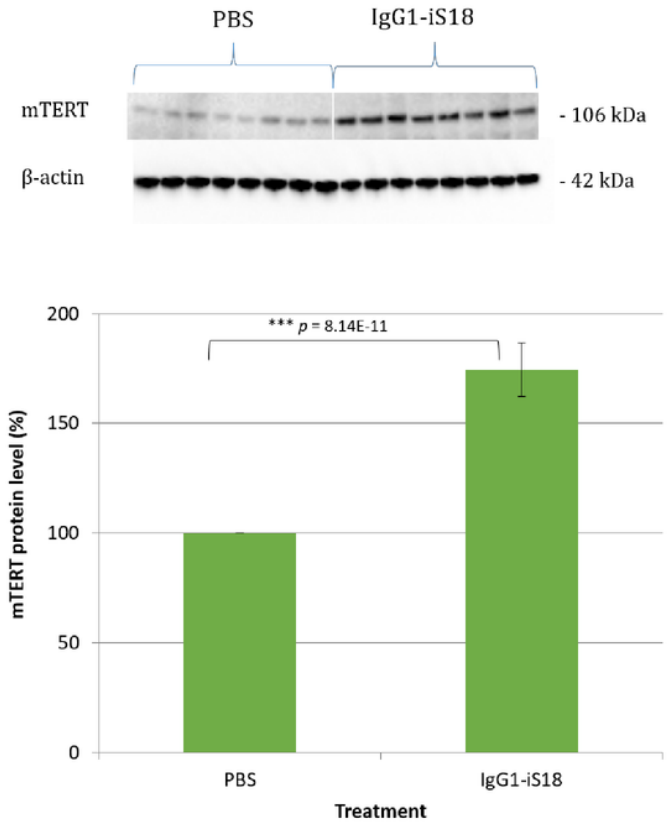

Fig. 9: Levels of mTERT in brain tissue of AD transgenic mice after treatment with IgG1-iS18 and PBS. Western blot analysis was performed and a significant increase in mTERT levels was observed after treatment with IgG1-iS18. Error bars represent standard deviation, n=8, *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (Adapted from Ferreira, Bignoux, Otgaar *et al.* Oncotarget, *in press*.

Upon performing a western blotting to detect mTERT levels, with subsequent densitometric analysis, it was revealed that mTERT levels in the antibody treated mouse brain samples were significantly higher (74.34%; p= 8.14E-11) on average, than the mTERT levels in the brains of the control mice (Fig. 9). This data, together with the reduction in amyloid plaque formation (Fig. 5), $A\beta_{42}$ levels (Fig. 6) and the increase in cognitive function (Fig. S1) in IgG1-iS18 treated mice, indicate a possible neuroprotective role of TERT.

Subsequently to having observed an increase in mTERT levels, qPCR analysis was performed to assess whether there was a difference in the activity of telomerase, since TERT is the catalytic subunit responsible for the reverse transcriptase activity of telomerase.

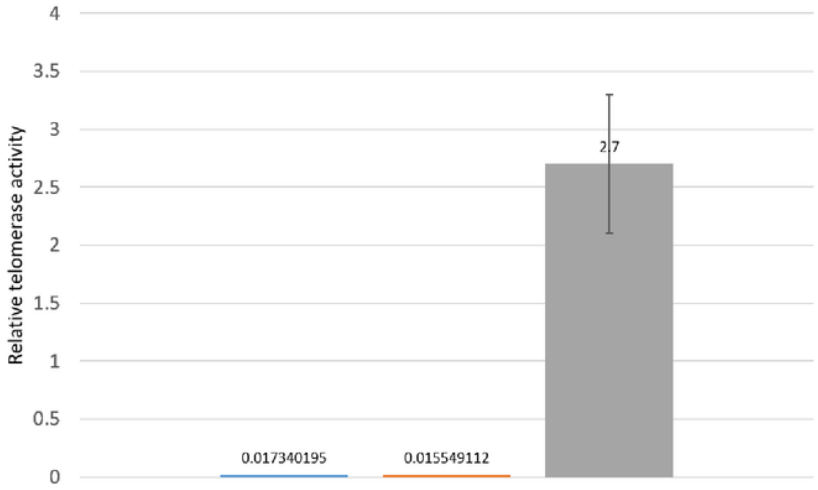

Fig. 10: Telomerase activity in mouse brains after treatment with PBS and IgG1-iS18. Telomerase activity in the mouse brains was compared to telomerase positive HEK293 cells and is almost negligible compared to the HEK293 control. No significant difference in telomerase activity in brains of AD transgenic mice was observed after treatment with IgG1-iS18. All values were normalised against the negative controls; all negative control values were subtracted from the signal of each sample. This data is representative of the mean value obtained across all biological repeats for each treatment and the HEK293 cells. Error bars represent standard deviation, n=8 per treatment group; n=6 for HEK293 samples. (Adapted from Ferreira, Bignoux, Otgaar *et al*. Oncotarget, *in press*).

Although TERT levels increased significantly, there was no significant difference observed in telomerase activity between the two treatment groups (Fig. 10). In fact, telomerase activity in the brains of the mice was almost negligible when compared to the telomerase positive, HEK293 cells. This is conducive to reports showing that adult mouse brains lack telomerase activity (Klapper & Mattson, 2001)

Since we know that DNA damage plays a key role in the aging process and that it is proposed there is a possible involvement of a DNA repair defect in the pathogenesis of AD (Coppede & Migliore, 2009), we decided to further validate the neuroprotective role of IgG1-iS18 and

assess the resultant effect on the DNA damage response by investigating the levels of phosphorylated H2AX (γH2AX). These histones are phosphorylated at Ser139 at the occurrence of DNA double stranded breaks and serve to marks sites of DNA damage as well as to aid in the recruitment of DNA repair factors (Odell *et al*., 2013). Therefore, we performed western blotting and subsequent densitometric analysis on the levels of γH2AX in the extracted samples from the hemi-brains of the IgG1-iS18 and PBS treated mice.

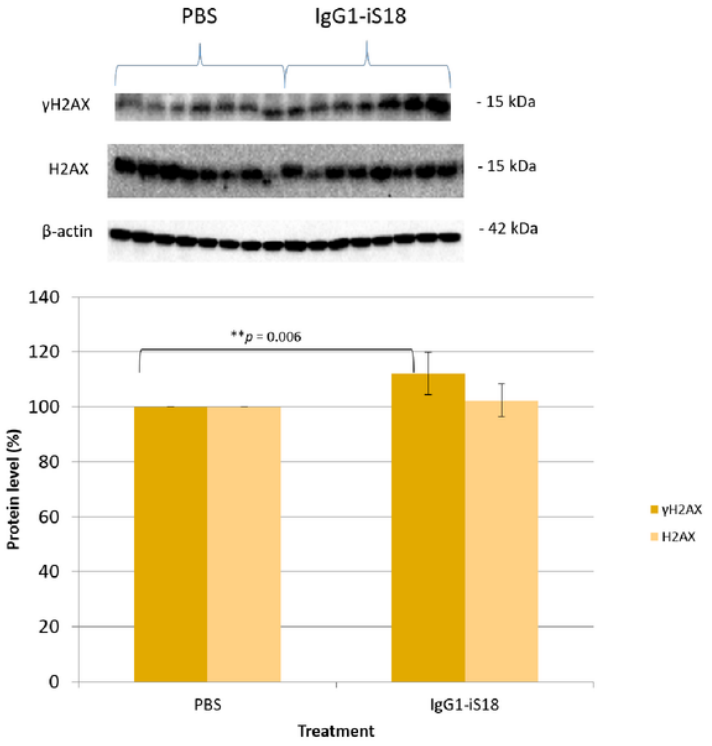

Fig. 11: Effect of IgG1-iS18 on phosphorylated (SER139) γH2AX protein levels in brain tissue of AD transgenic mice as detected by western blot analysis. A significant increase in PSER139 γH2AX levels was observed after treatment with IgG1-iS18. No significant difference is observed in H2AX levels. Error bars represent standard deviation, n=7. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test. (Adapted from Ferreira, Bignoux, Otgaar *et al*. Oncotarget, *in press*).

A significant increase (p=0.006) of 10% (on average), in the γH2AX levels in the antibody treated hemi-brain homogenates was observed compared to the PBS control treated mice (Fig. 11). Furthermore, there was no significant change in total H2AX levels between IgG1-iS18 treated mice and the control mice. Therefore, these results suggest an increase in the activation of the DNA repair process, reflected by the significant increase in γH2AX levels.

4.2. Alzheimer's disease in vitro study

Since it was suggested that an upregulation of TERT expression may be a possible strategy towards combatting AD (Wang *et al*., 2015) and that overexpression of LRP::FLAG causes an increase in hTERT expression (Otgaar *et al*.,2017), this part of the study focussed on stably transfecting HEK293 and SH-SY5Y cells with the pCIneo-moLRP::FLAG construct. Thereafter, the aim was to assess whether inducing overexpression of LRP::FLAG would offer a novel and potential therapeutic strategy for the treatment of AD. Refer to Fig. 12 for an overview of the design of the *in vitro* study.

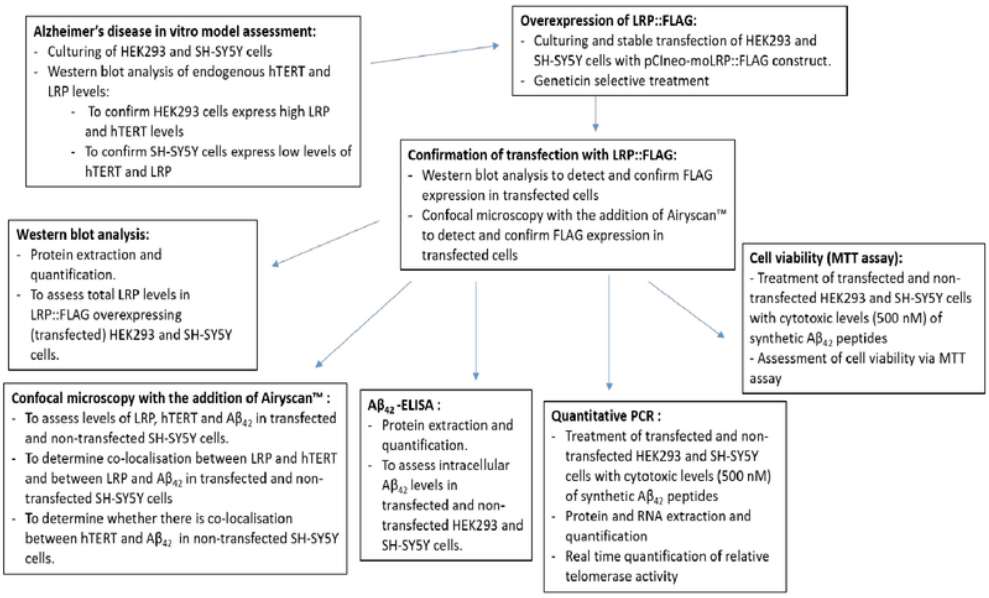

Fig. 12: Design of the Alzheimer's disease *in vitro* study. A brief outline of the experimental procedures performed to assess the effect of LRP::FLAG overexpression in HEK293 and SH-SY5Y Alzheimer's disease cell culture models.

4.2.1. Endogenous levels of LRP and hTERT in HEK293 and SH-SY5Y cells

Firstly, endogenous levels of LRP and hTERT were assessed via western blotting, in the SH-SY5Y *in vitro* AD model and compared to the respective levels in the telomerase/hTERT positive HEK293 control cells. This was to determine whether the SH-SY5Y cell line would provide a suitable model for the overexpression study.

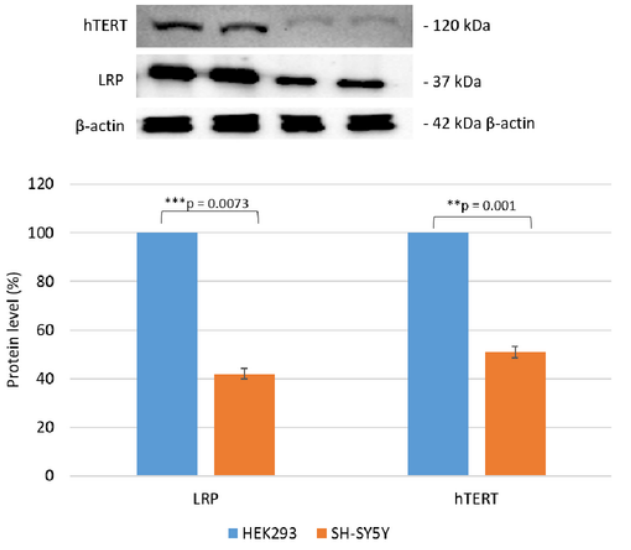

Fig. 13: Endogenous protein levels of LRP and hTERT in SH-SY5Y and HEK293 cells. Levels of LRP and hTERT are significantly lower in the SH-SY5Y cells when compared to HEK293 cells. Error bars represent standard deviation, n=3 biological repeats (only 2 repeats shown), *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

SH-SY5Y cells exhibit significantly lower endogenous levels of LRP (p=0.00729) and hTERT (p=0.001) levels compared to the respective levels in the HEK293 cells (Fig. 13). Thus, the study was continued with the use of the SH-SY5Y cells as the experimental cell line and the HEK293 cells as the TERT/telomerase positive control cell line.

4.2.2. LRP::FLAG overexpression significantly increases LRP levels

The SH-SY5Y cell line would provide a suitable model for overexpressing LRP::FLAG to assess the resultant effects of this potential therapeutic in an AD context. Therefore, the SH-SY5Y cells were transfected with the pCIneo-moLRP::FLAG construct and selectively treated for a period of 6 weeks to induce a stable transfection and overexpression of the LRP::FLAG protein. After this period, western blotting was used to detect whether the transfection was successful and thereafter to determine whether there was a subsequent increase in LRP levels in the HEK293 and SH-SY5Y cells.

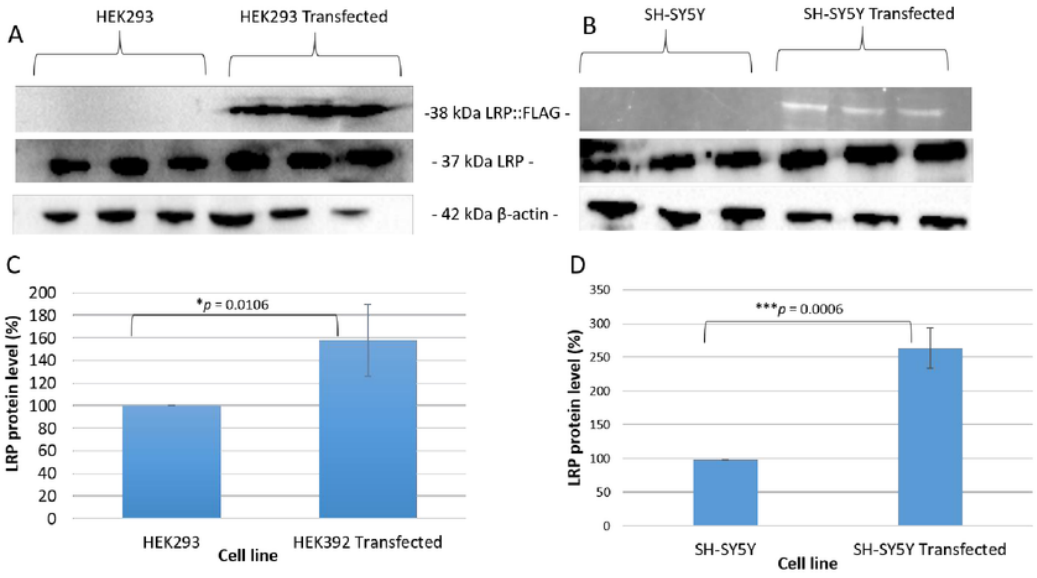

Fig. 14: Western blot analysis reveals that LRP::FLAG overexpression increases total LRP levels in HEK293 and SH-SY5Y cells. A significant increase in LRP levels was observed after stable transfection of HEK293 and SH-SY5Y cells with LRP::FLAG. (A) LRP::FLAG (HRP) is detected in HEK293 transfected cells and not in non-transfected HEK293 cells. LRP (HRP) expression is increased in transfected HEK293 cells. β-actin (HRP) is used as the loading control. (B) LRP::FLAG (Cy3) is detected in SH-SY5Y transfected cells and not in non-transfected SH-SY5Y cells. LRP (HRP) expression is increased in transfected SH-SY5Y cells. β-actin (HRP) is used as the loading control (C) Densitometric analysis indicates a significant 58 % increase in LRP protein level after LRP::FLAG transfection in HEK293 cells. (D) Densitometric analysis in SH-SY5Y transfected cells indicates LRP::FLAG overexpression significantly increases LRP protein levels by 165 %. Error bars represent standard deviation, n=3 biological repeats. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Here, it is shown that LRP::FLAG is exclusively expressed in the transfected HEK293 (Fig.14A) and SH-SY5Y (Fig. 14B) cells and not in the respective non-transfected cells. In addition, it was confirmed that LRP::FLAG overexpression did indeed increase total LRP protein levels. Moreover, upon densitometric analysis of the western blots comparing non-transfected and LRP::FLAG transfected cells, it was revealed that LRP::FLAG overexpression significantly increases LRP levels by 58 % (p= 0.0106) in HEK293 cells (Fig.14C) and by 165 % (p= 0.014) in SH-SY5Y cells (Fig. 14D).

4.2.3. Immunofluorescence microscopy with Airyscan™ reveals that overexpression of LRP::FLAG decreases Aβ levels with a concomitant increase in hTERT levels

Since it was shown via western blotting that the SH-SY5Y cells transfected with LRP::FLAG were indeed expressing LRP::FLAG and that there was a resultant increase in LRP levels, confocal microscopy was used to further confirm this. As such, confocal microscopy with the addition of Airyscan™ was used to firstly, confirm LRP::FLAG expression in the transfected SH-SY5Y cells and thereafter to assess the resultant effects on the levels and co-localisation of LRP, $A\beta_{42}$ and hTERT. Furthermore, this was not performed in the HEK293 cells as levels and localisation of these proteins were previously reported (Naidoo et al., 2015; Otgaar et al., 2017; Da Costa Dias et al., 2013).

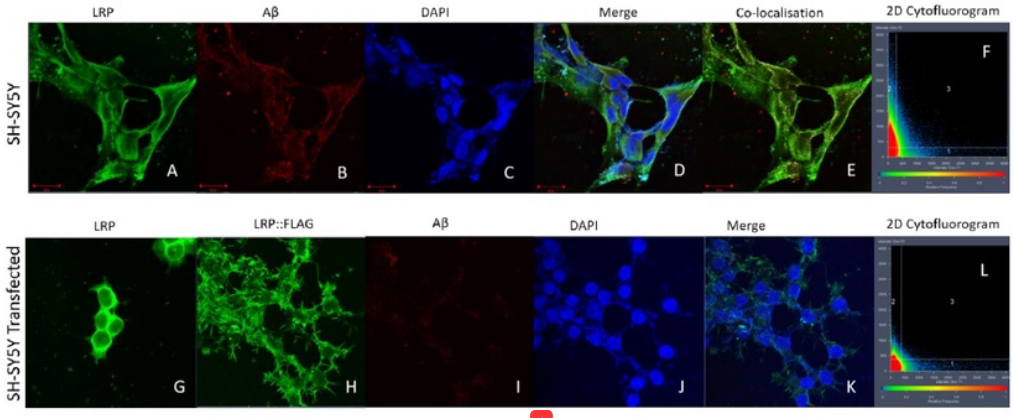

Fig. 15. LRP::FLAG overexpression decreases Aβ levels in SH-SY5Y cells. (A-L). Intracellular localisation and co-localisation of LRP (FITC-Green) and Aβ (APC-Red) as well as LRP::FLAG (FITC-Green) and Aβ in non- and LRP::FLAG transfected SH-SY5Y cells. (A). Endogenous LRP levels in SH-SY5Y cells, LRP localises to cytosol and cell surface. (B) Endogenous Aβ levels in SH-SY5Y cells, Aβ is localised to cytosol and cell surface. (G) LRP levels are increased in LRP::FLAG transfected cells. (H) LRP::FLAG expression is confirmed in transfected SH-SY5Y cells and localisation occurs predominantly on the cell surface and cytoplasm. (I) Aβ expression becomes almost undetectable upon overexpression of LRP::FLAG. (C&J) Nuclei are stained with DAPI [Blue]. Co-localisation occurs between LRP and Aβ both inside the cell and on the cell surface, represented by yellow fluorescence in merged images (D&K), as white areas (E) and as fluorescence in the third quadrant of the 2D cytofluorograms (F&L). All images are at 630 X magnification and a resolution of 140 nm. Scale bars represent 10 µm.

In non-transfected SH-SY5Y cells, LRP localises on the cell surface and intracellularly (Fig. 15A), as previously reported in (Mbazima *et al.*, 2010). Under the same conditions, Aβ tends

to localise to the cell surface as has been shown previously (Vetrivel &Thinakaran, 2010) and to a lesser extent in the cytoplasm (Fig.15B). Here, we see a low level of co-localisation (Fig. 15E) between LRP and Aβ on the cell surface and to a lesser extent in the cytoplasmic regions (Fig.15D-F). This is conducive to what was previously reported (Da Costa Dias et al., 2013). Upon confirmation of transfection of SH-SY5Y cells with LRP::FLAG (Fig. 15H) there is a clear increase in total levels of LRP (Fig. 15G). This is concomitant with a decrease in Aβ levels, whereby levels are almost undetectable (Fig. 15I) in comparison to the non-transfected cells (Fig.15B). See Fig. S2 (Appendix) for enlarged view of Fig. 15.

In addition, an interesting finding upon transfection is the morphological transformation that occurs (Fig. 15G-K; 15G-K), whereby cells change from an elongated epithelial-like morphology to a less-differentiated morphology with enlarged nuclei. Since this was not a focus of this study, this was not further examined. However, these changes are being investigated in a separate study.

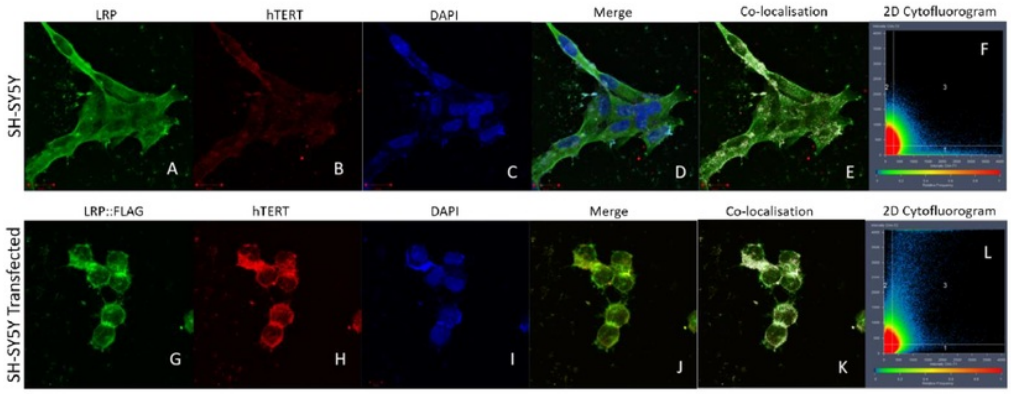

Fig. 16. LRP::FLAG overexpression increases hTERT levels in SH-SY5Y cells. (A-L). Intracellular localisation and co-localisation of LRP (FITC-Green) and hTERT (APC-Red) as well as LRP::FLAG (FITC-Green) and hTERT in non- and LRP::FLAG transfected SH-SY5Y cells. (A) Endogenous LRP levels in SH-SY5Y cells, LRP localises to cytosol, nucleus and cell surface. (B) Endogenous hTERT levels in SH-SY5Y cells, hTERT is localised to the nucleus and the cytosol. (C&I) Nuclei are stained with DAPI (Blue). (G) LRP levels are increased in LRP::FLAG transfected cells. (H) LRP::FLAG expression is confirmed in transfected SH-SY5Y cells. There is co-localisation between hTERT and LRP and this increases in SH-SY5Y cells overexpressing LRP::FLAG. This is represented by yellow fluorescence in merged images (D&J), as white areas (E&K) and as fluorescence in the third quadrant of the 2D cytofluorograms (F&L). All images are at 630 X magnification and a resolution of 140 nm. Scale bars represent 10 µm.

As aforementioned, LRP expression in non-transfected SH-SY5Y cells occurs widely throughout the cells (Fig. 16A). In these cells, hTERT localises to the nuclear and cytosolic regions (Fig. 16B). Here, co-localisation occurs between LRP and hTERT, as represented by yellow fluorescence (Fig. 16D-F). white areas. These results confirm previously reported results (Naidoo *et al*., 2015; Otgaar *et al*., 2017). However, upon transfection of the SH-SY5Y cells with LRP::FLAG (Fig. 16G), there is a clear increase in hTERT levels and intracellular localisation, as observed in Fig. 16H, which is furthermore shown by the increase in fluorescence intensity indicated in Fig. 15L. See Fig. S3 (Appendix) for enlarged view of Fig. 16.

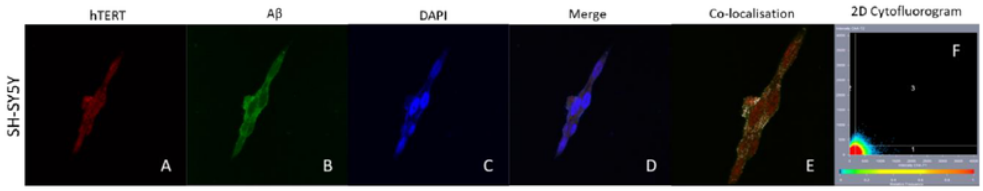

Fig. 17. Immunofluorescence microscopy in SH-SY5Y cells indicates co-localisation of hTERT and Aβ. (A-F) hTERT [APC-Red] (A) and Aβ [FITC-Green] (B) co-localise on the cell surface and intracellularly in SH-SY5Y cells. Nuclei are stained with DAPI [Blue] (C). Co-localisation is represented by yellow fluorescence in the merged image (D), as white areas (E) and as fluorescence in the third quadrant of the 2D cytofluorograms (F). All images are at 630 X magnification and a resolution of 140 nm.

Since we know of an antagonistic relationship between telomerase and Aβ in AD, as previously reported (Wang *et al*., 2015), confocal microscopy with the addition of Airyscan™ was employed to determine whether these two proteins co-localise in the cell. Here we observe that hTERT and Aβ do indeed co-localise within the cell as indicated by white areas in Fig. 17E and as detectable fluorescence in quadrant 3 of Fig. 17F. This furthermore suggests an interaction between the two proteins. Furthermore, this co-localization is indicative of a possible association between these proteins.

Co-localisation is presented as spatial overlap, indicated as yellow fluorescence in merged images (Fig. 15D, K; Fig. 16D, J; Fig. 17D). This is further presented by white areas, whereby, exact points of co-existence of the two relative proteins are seen (Fig. 15E; Fig. 16E, K; Fig. 17E). In addition, 2D cytofluorograms indicate fluorescence of both proteins being detected, whereby, FITC (Green) is shown in the 1st quadrant, APC (Red) is shown in

the 2nd quadrant and overlap between these fluorophores is seen in the 3rd quadrant (Fig. 15F, L; Fig. 16F, L; Fig. 17F). See Fig. S4 (Appendix) for enlarged view of Fig. 17.

4.2.4. LRP::FLAG overexpression significantly decreases intracellular Aβ42 levels in HEK293 cells

Since immunofluorescence microscopy indicated that SH-SY5Y cells transfected with LRP::FLAG exhibited lower Aβ42 levels (Fig.13I), an Aβ42-ELISA was performed on both the HEK293 and SH-SY5Y cells to further quantify this reduction.

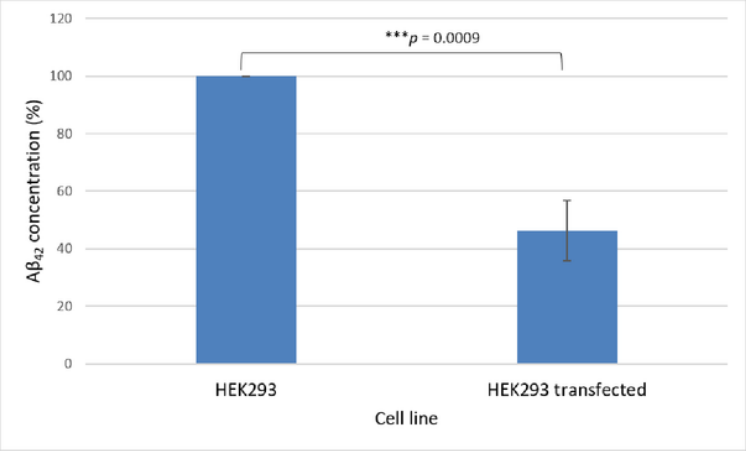

Fig. 18. LRP::FLAG overexpression decreases intracellular Aβ42 levels in HEK293 cells.
A significant decrease in total intracellular Aβ42 levels is observed in LRP::FLAG transfected cells. Aβ42 concentration is expressed as a % pg Aβ42 per mg total protein. Non-transfected HEK293 were set to 100 %. Error bars represent standard deviation, n=3 biological repeats. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Upon analysis of intracellular Aβ42 levels in the non-transfected and transfected HEK293 cells, it was observed that LRP::FLAG overexpression reduced these levels (Fig. 18). A significant reduction of 53.7 % in intracellular Aβ42 was observed in the LRP::FLAG transfected HEK293 cells, when compared to non-transfected HEK293 cells (p= 0.0009). Aβ42 levels were not detectable in non-transfected SH-SY5Y and LRP::FLAG transfected SH-SY5Y cells on analysis with the ELISA assay (Data not shown).

4.2.5. Overexpression of LRP::FLAG significantly increases telomerase activity in HEK293 and SH-SY5Y cells

Multiple studies have reported that an increase in TERT expression results in an increase in telomerase activity (Bodnar *et al*., 1998; Counter *et al*., 1998). Thus, subsequently to determining that LRP::FLAG overexpression increases hTERT levels (Fig. 16G), relative telomerase activity was quantified via qPCR. In addition, since $A\beta_{42}$ is known to be antagonistic with hTERT (Wang *et al*., 2015), telomerase activity was assayed in both the presence and absence of cytotoxic levels of $A\beta_{42}$ (Da Costa Dias *et al*., 2014) to determine whether the abovementioned factors influenced telomerase activity in both HEK293 and SH-SY5Y cells.

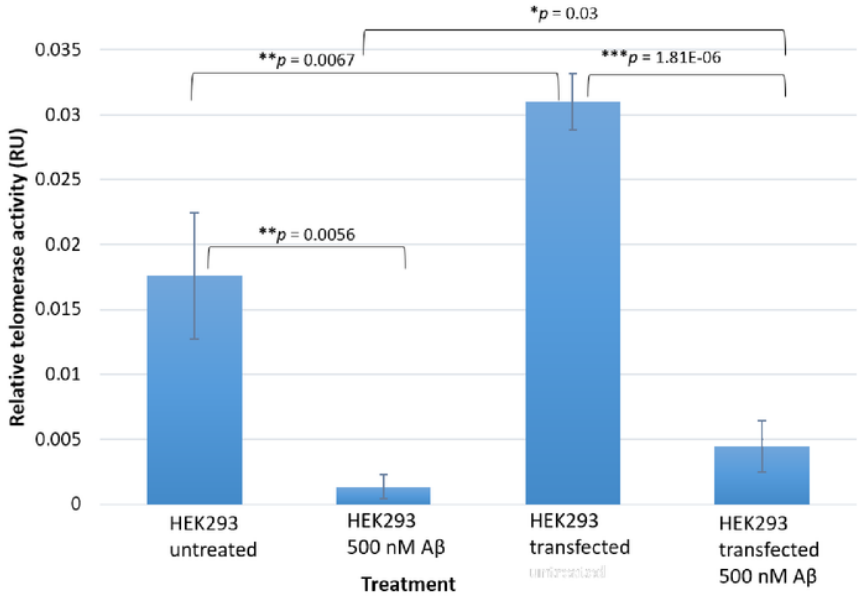

Fig. 19: LRP::FLAG overexpression significantly increases telomerase activity in HEK293 cells. Relative telomerase activity (RU= relative units) is increased in HEK293 and HEK293 cells transfected with LRP::FLAG after treatment with cytotoxic levels of synthetic $A\beta_{42}$ (500 nM). All values were normalised against the negative controls; all negative control values were subtracted from the signal of each sample. This data is representative of the mean value obtained across all biological repeats for each cell line and each treatment. Error bars represent standard deviation, n=3 biological repeats. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Upon relative quantification of telomerase activity in both untreated and $A\beta_{42}$ treated HEK293 and HEK293 transfected cells (Fig. 19), it was shown that indeed, LRP::FLAG overexpression increased telomerase activity. Furthermore, in the absence of $A\beta_{42}$ (0 nM),

HEK293 transfected cells had approximately 2-fold higher telomerase activity (p=0.0067) when compared to non-transfected HEK293 cells. This is conducive to what was previously reported (Otgaar *et al*., 2017). When these cells were treated with cytotoxic levels of synthetic Aβ$_{42}$, a significant decrease in the relative telomerase activity of both the non-transfected (p= 0.0056) and LRP::FLAG transfected (p= 1.81452E-06) HEK293 cells was observed. Interestingly, in the presence of cytotoxic levels of Aβ$_{42}$ (500 nM), HEK293 transfected cells exhibited significantly higher telomerase activity (p=0.03) in comparison to non-transfected HEK293 cells treated under the same conditions.

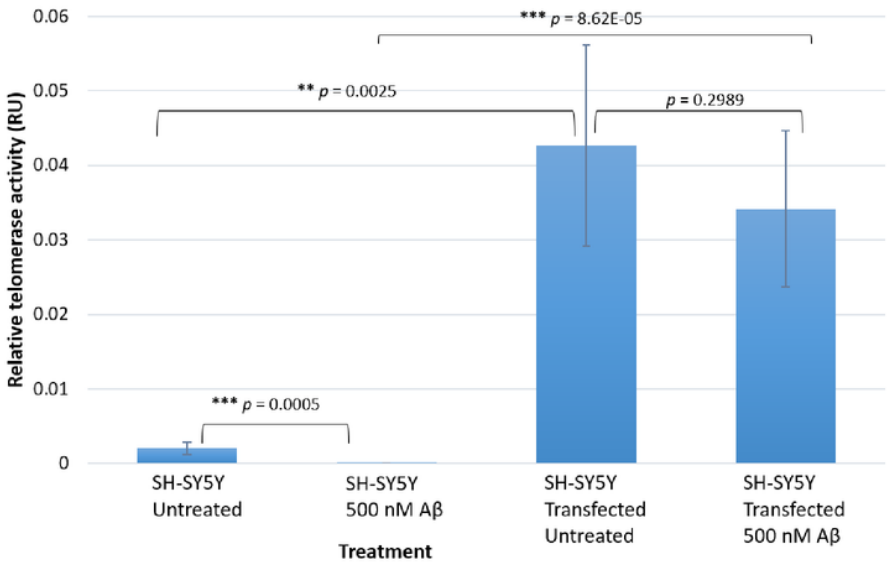

Fig. 20: LRP::FLAG overexpression significantly increases telomerase activity in SH-SY5Y cells. Relative telomerase activity (RU= relative units) is increased in SH-SY5Y and SH-SY5Y cells transfected with LRP::FLAG after treatment with cytotoxic levels of synthetic Aβ$_{42}$ (500 nM). All values were normalised against the negative controls; all negative control values were subtracted from the signal of each sample. This data is representative of the mean value obtained across all biological repeats for each cell line and each treatment. Error bars represent standard deviation, n=3 biological repeats. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Subsequent quantification of relative telomerase activity in the SH-SY5Y and SH-SY5Y transfected cells (Fig. 20) further indicated that LRP::FLAG overexpression results in an increase in telomerase activity. Upon assessment in non-transfected SH-SY5Y cells, very low

endogenous telomerase activity was observed. Moreover, upon treatment of these cells with cytotoxic Aβ42 levels, telomerase activity became almost negligible, with values similar to those of the negative controls. Additionally, in the absence of Aβ42, there was a significant increase in the relative telomerase activity in the SH-SY5Y cells overexpressing LRP::FLAG.This was in comparison to the non-transfected SH-SY5Ycells (p=0.00248) under the same conditions. Remarkably, when treated with 500 nM Aβ42, telomerase activity was significantly higher in the SH-SY5Y transfected cells when compared to the treated, non-transfected SH-SY5Y cells (p= 8.62407E-05).

4.2.6. Overexpression of LRP::FLAG rescues HEK293 and SH-SY5Y cells from Aβ42 – mediated cytotoxicity

LRP/LR is a known receptor for Aβ42, whereby it is involved in the internalisation and therefore, the accumulation of Aβ42 intracellularly, thereby inhibiting cell proliferation and inducing apoptosis (Da Costa Dias *et al*., 2014). In addition, treatment of cells with 500 nM synthetic Aβ42 peptides has proven to mimic Aβ42 – mediated cytotoxicity (Da Costa Dias *et al*., 2014). Since it was revealed that LRP::FLAG overexpression increased LRP and hTERT levels with a concomitant decrease in Aβ42 levels, an MTT assay was performed to determine whether there was an effect on the viability of the transfected HEK293 and SH-SY5Y cells in the presence of cytotoxic levels of synthetic Aβ42 peptides.

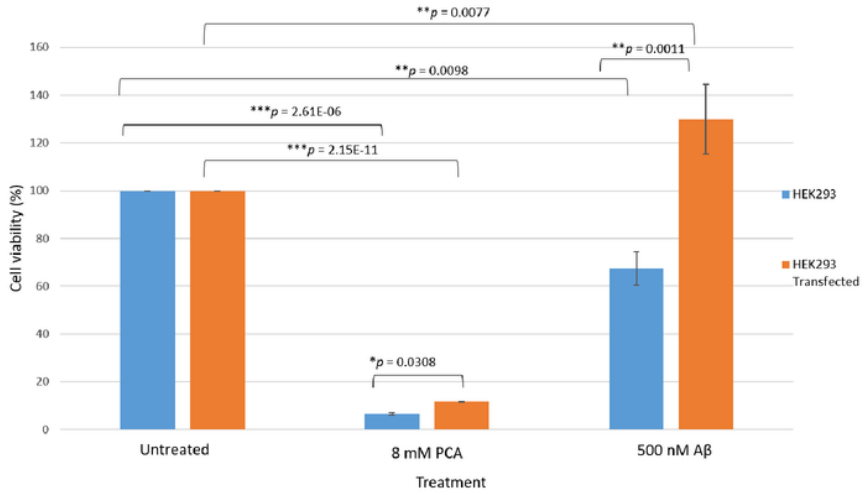

Fig. 21: LRP::FLAG overexpression rescues HEK293 cells from Aβ42 induced cytotoxicity. Cellular viability of HEK293 and HEK293 transfected cells, as determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay post exogenous treatment with

500 mM synthetic $A\beta_{42}$. Cell viability was assessed 48 h post-treatment and the untreated set to 100 %. Protocatechuic acid (PCA) was used as a positive control. Error bars represent standard deviation. n=3 biological repeats *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Analysis of cellular viability in HEK293 cells (Fig. 21) showed treatment with cytotoxic levels of $A\beta_{42}$ (to mimic $A\beta_{42}$-induced cytotoxicity) reduced cellular viability significantly in non-transfected HEK293 cells, with a decrease of 32.68% seen in comparison to untreated, non-transfected HEK293 cells (set to 100% cell viability) (p=00976) (Fig. 21). HEK293 cells treated with 8 mM of the apoptotic inducer, PCA, were used as a positive control and displayed 6.36% cell viability.

Interestingly, investigation of cell viability in HEK293 LRP::FLAG transfected cells (Fig. 21), 48 h post-treatment with 500 nM synthetic $A\beta_{42}$, revealed these cells exhibited a significant 62.62 % higher cell viability (p= 0.00105) when compared to non-transfected cells treated under the same conditions. Therefore, transfection with LRP::FLAG rescues HEK293 cells from $A\beta_{42}$- induced cytotoxicity.

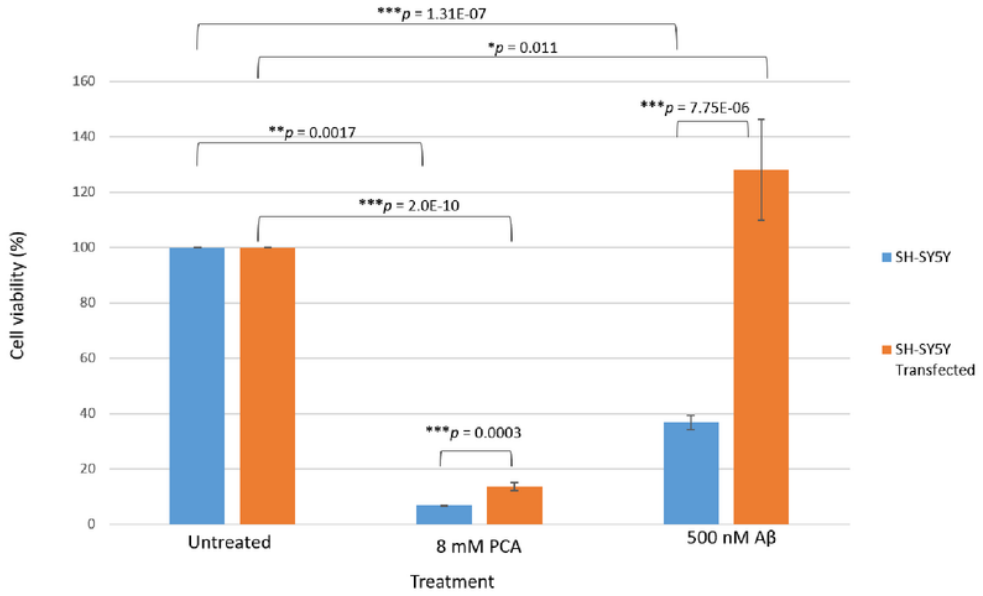

Fig. 22: LRP::FLAG overexpression rescues SH-SY5Y cells from $A\beta_{42}$ induced cytotoxicity. Cellular viability of SH-SY5Y and SH-SY5Y cells transfected with LRP::FLAG as determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay post exogenous treatment with synthetic $A\beta_{42}$. Cell viability was assessed 48 h post-treatment and the

untreated set to 100 %. PCA was used as a positive control. Error bars represent standard deviation. n=3 biological repeats. *p, 0.05, **p, 0.01, ***p, 0.001; Student's *t*-test.

Examination of cell viability in SH-SY5Y cells (Fig. 22) revealed that upon a 48-h treatment with 500 nM Aβ$_{42}$ synthetic peptides, there was a marked decrease of 63.33 % (p=1.31433E-07) in cell viability of the non-transfected SH-SY5Y cells. Whereby only 36.67 % of cells remained viable (untreated set to 100 %). However, when SH-SY5Y transfected cells were treated under the same conditions, they exhibited a significant 91.44 % higher viability in comparison to the treated, non-transfected cells. Non-transfected SH-SY5Y and LRP::FLAG transfected SH-SY5Y cells treated with 8 mM of the positive control, PCA, exhibited cellular viability of 6.76 % and 13.45 % respectively.

Taken together, these data indicate the novel finding that LRP::FLAG overexpression rescues both HEK293 and SH-SY5Y cells from Aβ$_{42}$-induced cytotoxicity.

5. Discussion

5.1. Alzheimer's disease transgenic mouse study:

Here, we establish the novel finding that treatment of 5XFAD transgenic mice through intranasal administration of the anti-LRP/LR specific antibody, IgG1-iS18, results in improved learning and short-term memory, decreased Aβ plaque formation and accumulation with a concomitant increase in mTERT levels. *in vitro* studies have shown that blockade of LRP/LR with IgG1-iS18 reduces Aβ shedding, decreases Aβ$_{42}$ internalization and increases cell survival in the presence of Aβ (Jovanovic *et al*., 2013; Da Costa Dias *et al*., 2013; Jovanovic *et al*., 2014). Thus, we hypothesized that administration of IgG1-iS18 through the nasal mucosa, might lead to reduction in Aβ generation and toxicity and therefore, mitigate symptoms associated with AD, including reduced cognitive function and Aβ plaque formation.

5.1.1. Intranasal administration of IgG1-iS18 decreases AD histopathological hallmarks and Aβ levels in brains of AD transgenic mice

In testing this hypothesis, Dr. Ferreira- van der Merwe reported that administration of IgG1-iS18 intranasally and biweekly for 8 weeks, significantly improved recognition and short-term memory (Fig S1). The novel object recognition test showed mice treated with the anti-LRP/LR specific antibody, IgG1-iS18, preferentially explored a novel object rather than a familiar object, therefore indicating an improvement in recognition memory. Furthermore, a

puzzle box test revealed that when mice were exposed to problem-solving tasks, IgG1-iS18 treated mice took significantly less time to reach the relevant goal zone, compared to the control. Therefore, indicating a significant improvement in short term and learning memory. The results from the novel object recognition and puzzle box tests were hereby reported in summary for contextual purposes as these tests were performed by Dr. Ferreira-van der Merwe. These data are extensively analysed and discussed in Ferreira, Bignoux, Otgaar, *et al*. Oncotarget, *in press.*

Since the memory tests provided positive results in the IgG1-iS18 treated mice, we decided to investigate whether this improvement in memory and cognitive function was due to an improvement in AD brain pathology. Therefore, histological analysis was performed to assess the effect on the β-amyloid load and its subsequent effects on tissue atrophy of the hippocampus of the AD mice. We observed a considerable decrease in vacuolization as well as Aβ plaque formation, key factors involved in causing neuronal loss and thus the behavioural and cognitive dysfunction experienced by AD sufferers (Serrano-Pozo *et al*., 2011; Xiao *et al*., 2015). Furthermore, upon quantification, the amyloid load was significantly lower in the hippocampus of the IgG1-iS18 treated mice when compared to that of the PBS treated mice (Fig 5). It has been established previously, that hippocampal lesions result in moderate memory impairment (Broadbent *et al*., 2010) and altered executive functions in the puzzle box test (Nada *et al*., 2011). Therefore, we suggest that there is a direct correlation between the enhancement in memory and cognitive function and the amelioration of the AD brain pathology.

To further substantiate the effect of nasal IgG1-iS18 treatment on Aβ levels, an $A\beta_{42}$ ELISA and dot-blot analysis were performed. This was to assess the Tris-soluble (soluble) and GuHCl-soluble (insoluble) $A\beta_{42}$ peptide levels, respectively, in the mouse hemi-brain homogenates from both treatment groups. Here, we demonstrated a significant decrease in both the soluble and insoluble $A\beta_{42}$, expressly in the IgG1-iS18 treatment group (Fig. 6). This coincides with our previously published findings, whereby, IgG1-iS18 treatment of HEK293 and SH-SY5Y cells lead to a decrease in Aβ levels (Jovanovic *et al*., 2013). Thus, we propose that the observations seen upon assessment of cognitive ability and brain pathology in the mice treated with the IgG1-iS18 antibody are causally related to the significantly lower $A\beta_{42}$ levels revealed explicitly in these mice.

5.1.2. IgG1-iS18 treatment significantly increases APP brain levels.

It has previously been reported that FRET analysis (Jovanovic *et al*., 2014) revealed a direct interaction between LRP/LR and γ-secretase and an indirect interaction between LRP/LR and β-secretase. Thereby, indicating that LRP/LR is involved in the APP cleavage process. Moreover, IgG1-iS18 blockade of LRP/LR impedes these interactions (Jovanovic *et al*., 2015). Thereby, preventing the formation and shedding of $A\beta_{1-42}$ by inhibiting the sequential cleavage of APP by the β- and γ-secretases (Jovanovic *et al*., 2013) without affecting the levels of these AD related proteins. Considering these formerly attained *in vitro* results, we wanted to confirm whether the IgG1-iS18 antibody treatment had no effect on the levels of LRP/LR, β- and γ-secretase as well as APP in the mouse brain tissue of these mice when compared to the PBS treated control mice. Western blot analysis indeed showed uniform levels of LRP/LR, β- and γ-secretase expression among the mice (Fig. 7). Therefore, this is suggestive that treatment with IgG1-iS18 does not modulate the gene-expression of these AD related proteins. Interestingly, in contrast to what was previously ascertained *in vitro*, we observed that treatment with the anti-LRP/LR antibody resulted in a significant increase of 85.86 % in APP levels in these mice (Fig. 8). Therefore, we suggest that this increase in APP levels is caused by the inhibition of the APP cleavage process, due to the diminished interaction between LRP/LR, β- and γ-secretase (Jovanovic *et al*. 2015). Furthermore, this occurs concomitantly with the observed reduction in $A\beta_{1-42}$ production (Fig. 6).

5.1.3. Intranasal IgG1-iS18 treatment significantly increases mTERT levels and phosphorylation of H2AX

In addition to the abovementioned interactions between LRP/LR, β- and γ-secretase (Jovanovic *et al*., 2014), it was recently elucidated that LRP/LR co-localizes with the reverse transcriptase ribonucleoprotein, TERT, in both tumorigenic and non-tumorigenic cells (Naidoo *et al*., 2015; Otgaar *et al*., 2017). Furthermore, the interaction between these proteins was confirmed by co-immunoprecipitation assays (Naidoo *et al*., 2015). In addition to the role of TERT as the reverse transcriptase ribonucleoprotein in telomerase, TERT further performs non-telomeric functions. It has been reported that TERT plays a protective function against neuronal apoptosis caused by various stresses (Li *et al*., 2013) and furthermore, is involved in DNA damage responses and repair (Saretzki *et al*., 2014). It is recognized that the occurrence of and predisposition to AD, increases with age. This is supported by evidence

showing that AD sufferers present shorter telomere lengths in their neuronal cells (Wang *et al*., 2015), a key factor in cellular senescence and the aging process (Shay & Wright, 2005).

Moreover, it has recently been reported that the pathological mechanisms of AD are related to human telomerase. Wang *et al*., 2015 illustrated in an *in vitro* setting, that $A\beta_{1-40}$ and $A\beta_{1-42}$ inhibit telomerase activity by means of binding to telomeric DNA/RNA complexes of telomerase. Furthermore, Zhu *et al*., 2000 had previously established a neuroprotective function of TERT in an *in vitro* AD experimental model. Whereby, they reported an increase in vulnerability to Aβ-induced apoptosis upon downregulation of TERT and inhibition of telomerase activity in embryonic hippocampal neurons. Moreover, when they overexpressed TERT in this *in vitro* AD experimental model, they reported an increase in antiapoptotic responses. Altogether indicating that telomerase has a function in promoting cell survival (Zhu *et al*., 2000). Thus, we performed western blotting to establish if the observed improvement in cognitive ability and decrease in Aβ levels and plaque formation seen after antibody treatment, was accompanied by a simultaneous increase in mTERT levels. Interestingly, we revealed a significant increase in mTERT protein levels in the hemi-brain homogenates of the IgG1-iS18 treated mice when compared to the levels in the brain tissue of the PBS treated control (Fig. 9). Therefore, we propose that the observed decrease in Aβ levels, after treatment with IgG1-iS18, resulted in a reduction in neurotoxicity and hence a concomitant increase in mTERT levels. It is also possible that this increase in mTERT levels played an independent and additional neuroprotective role against the Aβ-induced neurotoxicity. This is suggested as TERT is known to have a protective role in the mitochondria, whereby TERT translocates to the mitochondria in the presence of ROS and aids in the protection of the organelle against mtDNA damage and apoptosis (Cong & Shay, 2008). This is further suggested, as it is known that Aβ neurotoxicity is related to Aβ-induced neuronal apoptosis, which is primarily responsible for mitochondrial dysfunction (Mattson, 1997) and DNA damage (Zhang *et al*., 1995; Deng *et al*., 1999), which are prevalent in AD sufferers.

Since we observed an increase in mTERT levels and reports have shown that increases in TERT expression result in an increase in telomerase activity (Bodnar *et al*., 1998; Counter *et al*., 1998), we wanted to determine whether treatment with IgG1-iS18 influenced telomerase activity in the mouse brain tissue. We detected very low levels of telomerase activity with no significant change between control and treated mice (Fig 9) and therefore suggest that the increased levels in TERT expression observed in the mouse brain, have a neuroprotective

function against age-related and Aβ-induced neurodegeneration rather than playing a role in telomerase activity. Furthermore, studies have reported that adult mouse brains lack telomerase activity (Klapper *et al*., 2001)

As aforementioned, Aβ neurotoxicity is associated with Aβ induced neuronal apoptosis. This occurs via oxidative stress and disrupted cellular calcium homeostasis, both of which, contribute to the aforestated DNA damage (Zhang *et al*., 1995; Deng *et al*., 1999) and mitochondrial dysfunction (Mattson, 1997). There are numerous factors involved in the DNA repair process as well as in signalling the presence of damage, which have shown to accumulate after double-strand DNA breaks. One such important response to DNA damage is the phosphorylation of H2AX (γH2AX) (Paull *et al*., 2000). This facilitates the recruitment of a subset of damage response and repair proteins. Masutomi *et al*., 2005 revealed that the DNA damage response was impaired in cells lacking hTERT. Moreover, this was due to a reduction in the phosphorylation of H2AX, thus implicating hTERT as a crucial component of the DNA damage response pathway. Subsequent to observing a decrease in neurotoxicity and an increase in mTERT levels after treatment with IgG1-iS18, we decided to investigate the levels of γH2AX present in the brain tissue. Surprisingly, the antibody treatment significantly increased levels of γH2AX by 10 %, whilst total H2AX levels remained unchanged (Fig. 11). It is possible that the increase in the phosphorylation of H2AX was due to the concomitant increase in mTERT and might contribute to the repair and protection against neurodegeneration as observed in this study.

Therefore, intranasal administration of IgG1-iS18 improved cognitive function, reduced amyloid plaque formation and $A\beta_{42}$ levels with a concomitant increase in APP and mTERT levels, together with an increase in H2AX phosphorylation. Ultimately, IgG1-iS18 prevents the pathological progression of AD in AD transgenic mice.

5.2. Alzheimer's disease in vitro study:

The second part of this study involved an investigation into the effect of overexpressing LRP::FLAG on LRP, TERT and Aβ protein levels, as well as the resultant effect on telomerase activity and cell viability in the HEK293 and SH-SY5Y AD cell culture models. Here, the respective protein levels were assessed by western blotting and confocal microscopy with the addition of Airyscan™. Thereafter, $A\beta_{42}$ levels were assessed by ELISA, telomerase activity via qPCR and cell viability by MTT analysis.

5.2.1. LRP::FLAG overexpression increases LRP and hTERT levels and diminishes Aβ production.

HEK293 and SH-SY5Y cells are routinely used for neurological studies for their expression of neuronal markers and ease of transfection (for review see Schlachetzki *et al*., 2013). It has previously been reported that inducing overexpression of LRP::FLAG via stable transfection, with the pCIneo-moLRP::FLAG construct, increased TERT levels, telomere length and telomerase activity in HEK293 cells (Otgaar *et al*., 2017). Since HEK293 cells express relatively high levels of endogenous TERT and have detectable telomerase activity, these cells were used as a positive control. Western blotting was performed to assess endogenous levels of LRP and hTERT in the SH-SY5Y cells and compared to the HEK293 control line (Fig. 13). Here, it was shown that LRP and hTERT levels are significantly lower in the SH-SY5Y cells in comparison to the HEK293 cells. HEK293 cells are reported to express relatively high levels of hTERT (Saretzki, 2014). Whereas, the SH-SY5Y are a somatic cell line of neuronal origin (Kovalevich & Langford, 2013) and mature neurons are reported to have low levels of endogenous TERT (Fu *et al*., 2000). Therefore, the SH-SY5Y cells were used as the experimental cell line. Thus, the HEK293 and SH-SY5Y cells provide a suitable *in vitro* model for LRP::FLAG overexpression, in order to study aspects of AD in cells with high and low telomerase activity, respectively.

Therefore, the SH-SY5Y and HEK293 cells were stably transfected with the pCIneo-moLRP::FLAG construct to induce overexpression of the LRP::FLAG protein. Confocal microscopy with the addition of Airyscan™ was performed in the SH-SY5Y cells to investigate the resultant effect of LRP::FLAG overexpression on LRP and hTERT levels, as well as co-localisation between these proteins. It was previously reported that LRP/LR both co-localises and interacts with hTERT in HEK293 and MDA_MB231 cells (Naidoo *et al*., 2015). Here, it is seen that LRP and hTERT do indeed co-localise in the SH-SY5Y cells (Fig. 16D-F) and that co-localisation occurs in the nuclear and cytosolic regions. In addition, confocal microscopy with Airyscan™ further reveals an increase in LRP (Fig. 16G) and hTERT (Fig. 16H) levels. Additionally, analysis via western blotting revealed that LRP::FLAG overexpression significantly elevated LRP levels in both the HEK293 (Fig. 14A, C) and SH-SY5Y (Fig. 14B, D) cells, by 58% and 165% respectively. Since an increase in hTERT levels was observed with confocal microscopy, western blotting with subsequent densitometric analysis will further be performed to provide a more accurate quantification of these levels. Since co-localisation studies between LRP/LR and hTERT and LRP/LR and

Aβ42, have been previously reported in HEK293 cells (Da Costa Dias *et al*., 2013; Otgaar *et al*., 2017) confocal microscopy was not performed in this cell line.

LRP/LR is a receptor of Aβ42 (Da Costa Dias *et al*., 2013) and is known to be involved in Aβ42 shedding (Jovanovic *et al*., 2013) and internalisation (Jovanovic *et al*., 2014). Thus, it was investigated whether there was co-localisation between LRP and Aβ42 in the SH-SY5Y cells. Indeed, it was observed that LRP and Aβ42 co-localise on the cell surface, as previously shown upon cell surface analysis in HEK293 cells (Da Costa Dias *et al*., 2013). Interestingly, co-localisation was also observed in the cytosol. This is conducive to reports indicating that Aβ42 incorporates into cell organelles (Verdier & Penke, 2004) as well as reports concluding LRP/LR localises in the cytoplasm, where it plays a role in translational processes (Jovanovic *et al*., 2015). However, upon transfection with LRP::FLAG, Aβ42 levels are diminished (Fig. 15I). This was a promising observation, considering Aβ42 is the known aetiological agent of AD (Choi *et al*., 2014), due to its role in the formation of amyloid plaques

Confocal microscopy with Airyscan™ indicated a decrease in Aβ42 levels (Fig. 15) in the LRP::FLAG transfected cells. Thus, an Aβ1-42- ELISA was performed to provide a more quantitative measure of intracellular Aβ42 levels. As anticipated, there was a significant decrease observed in intracellular Aβ42 levels in the HEK293 transfected cells, when compared to the non-transfected HEK293 cells (Fig. 18). However, on assessment in SH-SY5Y cells, Aβ42 levels were undetectable with the use of the Aβ1-42- ELISA. We suggest this is as these cells were not fully differentiated to the more mature neuron-like phenotype and as such do not express all neuronal markers (Kovalevich &Langford, 2013). Since we saw an increase in LRP and hTERT protein levels, we propose this concomitant decrease in Aβ42 levels seen in both the SH-SY5Y (Fig. 15) and the HEK293 (Fig. 18) cells is owed to the antagonistic relationship between telomerase and Aβ in AD (Wang *et al*., 2015). The observed decrease in intracellular Aβ42 levels could be indicative of a decrease in Aβ42 internalisation. This will be further assessed by forming an Aβ42-ELISA in the transfected and non-transfected HEK293 and SH-SY5Y cells, which have been treated with cytotoxic levels (500 nM) Aβ42.

Since it was previously observed that LRP co-localises with hTERT and with Aβ42, respectively, it was of interest to assess whether hTERT and Aβ42 co-localise. Indeed, confocal microscopy with Airyscan™ analysis revealed co-localisation between hTERT and Aβ42 occurs within SH-SY5Y cells in the cytoplasm (Fig. 17). Wang *et al*., 2015 proposed

that there is an antagonistic relationship between telomerase and Aβ42 within neurons since Aβ42 is involved in the inhibition of telomerase activity, through the binding of the telomeric DNA/RNA template of telomerase (Wang *et al*., 2015). This, together with the results observed in the previous study strongly suggests there might be a direct interaction between these proteins. However, FRET analysis will be performed to confirm this. Altogether, overexpression of LRP::FLAG increases in LRP (Fig. 15G, 14G; Fig. 14) and hTERT levels (Fig. 16H) with a concomitant decrease in Aβ42 levels (Fig. 15I; Fig. 20).

5.2.2. LRP::FLAG overexpression elevates telomerase activity.

It has been reported by multiple studies that an increase in hTERT expression causes an increase in telomerase activity and that TERT behaves as the limiting factor for telomerase activity (Bodnar *et al*., 1998; Counter *et al*., 1998). Therefore, as we observed an increase in hTERT levels in the pCIneo-moLRP::FLAG transfected cells, we wanted to determine whether there was a resultant increase in telomerase activity. Since we know telomerase activity and Aβ42 have an antagonistic relationship (Wang *et al*., 2015) and that overexpression of LRP::FLAG decreased Aβ42 levels (Fig. 16; Fig. 18), we, therefore wanted to further determine the resulting effect on telomerase activity in both the absence and presence of cytotoxic levels of Aβ42. Hence, qPCR quantification of relative telomerase activity was performed in the HEK293 and SH-SY5Y cells. Here, it was confirmed, that there was indeed a significant increase in telomerase activity in both HEK293 (Fig. 19) and SH-SY5Y (Fig, 18) cells, after transfection with LRP::FLAG. Since there was an observable increase in hTERT levels after LRP::FLAG transfection, we expected to see an increase in telomerase activity, since this follows the trend as previously reported on in HEK293 cells (Otgaar *et al*., 2017). Additionally, non-transfected HEK293 cells exhibited higher basal levels of telomerase activity, when compared to the non-transfected SH-SY5Y cells. This is expected as HEK293 cells exhibited higher endogenous hTERT levels (Fig. 16). Although telomerase expression is a known hallmark of cancer, it has been shown that ectopic expression of telomerase is not associated with malignancy (Morales *et al*., 1999).

In addition, treatment with cytotoxic levels of Aβ42, thereby mimicking Aβ42-mediated cytotoxicity, caused a consequential reduction in telomerase activity in both transfected and non-transfected HEK293 and SH-SY5Y cells. Since we know Aβ42 induces apoptosis (Zhu *et al*., 2000) and inhibits telomerase activity (Wang *et al*., 2015), it is proposed that this reduction in telomerase activity is observed as a combination of these two factors.

Remarkably, however, telomerase activity was significantly higher in both the HEK293 (Fig. 19) and SH-SY5Y (Fig. 20) transfected cells when treated with synthetic $A\beta_{42}$ peptides and compared to their non-transfected counterparts treated under the same conditions. Thus, this novel finding suggests overexpression of LRP::FLAG significantly increases telomerase activity, even in the presence of $A\beta_{42}$. This observation is proposed to be attributable to the fact that the increase in hTERT levels and the resulting increase in telomerase activity after overexpression of LRP::FLAG, is occurring prior to the manifestation of $A\beta_{42}$-mediated cytotoxicity. Therefore, the overexpression of hTERT can compensate for the adverse effects caused by the high levels of $A\beta_{42}$. We suggest, that since telomerase activity is increased in the LRP::FLAG transfected cells, that there is likely an increase in telomere length, as previously reported (Otgaar *et al*., 2017). However, this will be assessed by qPCR.

5.2.3. Overexpression of LRP::FLAG rescues HEK293 and SH-SY5Y cells from $A\beta_{42}$-mediated cytotoxicity

LRP/LR has been shown to be a receptor for synthetic $A\beta_{42}$ peptides (Da Costa Dias *et al*., 2013) and is involved in the internalisation and subsequent accumulation of $A\beta_{42}$ intracellularly (Jovanovic *et al*., 2014). This results in $A\beta_{42}$-mediated cytotoxicity (Da Costa Dias *et al*., 2013). Furthermore, when cells are treated with 500 nM synthetic $A\beta_{42}$ peptides, thereby mimicking $A\beta_{42}$-mediated cytotoxicity, cell viability is decreased without affecting cell proliferation (Da Costa Dias *et al*., 2013). As we observed a reduction in $A\beta_{42}$ levels (Fig. 15; Fig. 18) together with an increase in LRP (Fig. 14; Fig. 15) and hTERT protein levels (Fig. 16), as well as telomerase activity (Fig. 19-18), we wanted to determine whether there was a resultant increase in cell viability in the presence of exogenous $A\beta_{42}$ peptides. Thus, an MTT assay was performed on both the transfected and non-transfected HEK293 and SH-SY5Y cells, including a 48-h exogenous treatment with 500 nM synthetic $A\beta_{42}$ peptides. Interestingly, less cell death was observed in both the HEK293 (Fig. 21) and the SH-SY5Y (Fig. 22) transfected cells, post exogenous treatment with cytotoxic levels of $A\beta_{42}$ peptides, when compared to the respective non-transfected cells. Therefore, indicating that LRP::FLAG overexpression rescues cells from Aβ-mediated cytotoxicity. We observed an increase in hTERT levels and telomerase activity concomitantly with a decrease in intracellular $A\beta_{42}$ levels and an increase in cell viability of LRP::FLAG expressing cells, in the presence of cytotoxic $A\beta_{42}$ levels. It is suggested that the mechanism by which hTERT interrupts the apoptotic cascade is attributed to the role of hTERT in the addition of TTAGGG repeats. This is consistent with the ability of telomerase inhibitors to induce

apoptosis (Chin *et al*., 1999; Fu *et al*., 1999), as well as the ability of telomerase to increase cell viability, as presented in this study. Furthermore, as ascertained in this study, hTERT localizes to both the nuclear and cytosolic regions (Fig. 16). Fu *et al*., 2000 elucidated that TERT can prevent the activation of caspases, before mitochondrial dysfunction can occur. TERT is reported to interact with the tumour suppressor, p53, through an interaction with the TERT-binding protein, TEP-1 (Li *et al*., 1999a). The pathological agent of AD, Aβ, has a functional role in mitochondrial dysfunction by disrupting calcium homeostasis through the formation of ion-permissible channels in the cell membrane, as well as causing oxidative stress (Mattson, 1997). In addition, ROS-induced alterations in mitochondrial membrane potential have been shown to be caused by p53, thereby, promoting apoptosis (Li *et al*., 1999b). It is reported that p53 is present at elevated levels in neurons affected by amyloid plaques (de la Monte *et al*., 1997), as well as in transgenic mice overexpressing the Aβ peptide (LaFerla *et al*., 1996). Therefore, it is likely that TERT is furthermore exerting a protective effect in the cytoplasm, against the aforementioned detrimental effects of Aβ, through the modulation of p53-dependent mechanisms. Thus, the data acquired in the present study are consistent with this possibility and as such, we suggest TERT is playing a protective function against apoptotic stresses caused by Aβ and is thereby, providing a neuroprotective function. However, to confirm these effects, Annexin V and caspase assays will be performed.

5.2.4. Conclusion.

The pathological process of AD has been attributed to the accumulation and aggregation of $A\beta_{42}$ (Choi *et al*., 2014), which occurs due to the abnormal proteolytic processing of APP, via the amyloidogenic pathway (Caetano *et al*., 2011). Furthermore, it is known that this accumulation promotes neuronal apoptosis as it mediates oxidative stress, mitochondrial dysfunction, DNA damage and caspase activation (Mattson, 1997; Zhang *et al*, 1995; Deng *et al*, 1999; Loo *et al*., 1993). It is suggested that the mechanism by which Aβ causes these effects, is either through direct interaction with cell surface receptors (Da Costa Dias *et al*., 2011) or by incorporating into lipid membranes and cell organelles (Verdier & Penke, 2004). LRP/LR is a known receptor of $A\beta_{42}$ and is furthermore involved in the shedding and internalisation of this peptide (Da Costa Dias *et al*., 2013; Jovanovic *et al*., 2014). Telomerase, the reverse transcriptase ribonucleoprotein responsible for telomere elongation and maintenance (Greider & Blackburn, 1987; Kim *et al*., 1994) is involved in AD. More specifically, AD sufferers present with shorter telomeres (Franco *et al*., 2006), suggestive of a

decrease in telomerase activity. Furthermore, TERT, provides extra-telomeric functions principally for the conservation of cell viability (Saretzki, 2014) and proliferation (Choi *et al.*, 2008; Cong & Shay, 2008; Park *et al.*, 2009). Moreover, it has been reported that TERT can protect neurons against Aβ-induced apoptosis (Zhu *et al.*, 2000) through its role in protecting against mtDNA damage and DNA repair (Mattson, 1997; Zhang *et al*, 1995; Deng *et al*, 1999).

The first part of this study is an original report proposing that intranasal administration of the anti-LRP/LR antibody, IgG1-iS18, decreases levels of soluble and insoluble $A\beta_{42}$ in the whole brain and impedes accumulation of amyloid plaques in the hippocampus. We suggest that the concomitant increase in mTERT levels and H2AX phosphorylation lead to a decrease in neurotoxicity and accordingly conveyed improvement of cognitive abilities, learning and short-term memory. Therefore, we endorse the anti-LRP/LR specific antibody, IgG1-iS18, as a novel and powerful potential therapeutic strategy for treatment of AD. This therefore, motivates the employment of clinical studies to further investigate the effect of IgG1-iS18 on patients suffering from AD. The second part of this study has shown that LRP::FLAG overexpression increases hTERT expression, concomitantly increases telomerase activity, and concurrently decreases intracellular $A\beta_{42}$ levels in HEK293 and SH-SY5Y AD cell culture models. We therefore propose that LRP::FLAG overexpression is providing a neuroprotective role as indicated by the rescue from $A\beta_{42}$-induced cytotoxicity observed in this study. Thus, overexpression of LRP::FLAG intracellularly, is a potentially powerful therapeutic for the treatment of AD.

Taken altogether, IgG1-iS18 blocks internalisation and shedding of $A\beta_{42}$, subsequently increasing mTERT protein levels, whilst overexpressing LRP::FLAG intracellularly, increases hTERT levels and consequently decreases $A\beta_{42}$ production. Both treatment strategies are therefore, allowing TERT to provide a neuroprotective role in an AD setting. Therefore, this prompts the implementation of an AD transgenic mice study. Whereby a combinatorial treatment can be employed, by which IgG1-iS18 is adopted to block the internalisation and shedding of $A\beta_{42}$. Whilst overexpression of LRP::FLAG intracellularly is applied to reduce $A\beta_{42}$ production simultaneously. If the transgenic mouse study provides positive results, the study can proceed to clinical trials, whereby the treatments can be administered as protein-based drugs. Ultimately, Targeting LRP/LR in an AD context has provided insight into the disease and simultaneously presented alternative therapeutic options for halting disease progression in those suffering from AD.

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---

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