Alternative splicing and its contribution to cognitive decline in the ageing brain

Submitted by Jed Lye to the University of Exeter as a thesis for the degree of Master's by Research in Medical Sciences

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I certify that all material which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other university.

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ABSTRACT

Premature cognitive decline is a feature of age-related pathological processes, often associated with neurodegenerative disorders. Part of the systemic ageing process is the accumulation of senescent cells in tissues around the body, including the brain. Cellular senescence contributes to the systemic inflammation which comes with ageing and disrupts several cellular regulatory mechanisms. Alternative splicing is one of these processes. Here, we first characterise the inflammatory component or senescence associated secretory phenotype and the splicing dysregulation within astrocytes. We then observe and quantify the effects of this on the alternative splicing of essential transcripts related to astrocyte function. Finally, we assess the correlation between observable levels of splicing perturbation in peripheral whole blood and the progression of cognitive decline in a population study. As a result, we identify potential biomarkers for the cognitive decline associated with dementia and other neurodegenerative disorders.

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Abbreviations

AKAP17A ..... A-Kinase Anchoring Protein 17A

AQP4.....Aquaporin 4

CDKN2A..... Cyclin-Dependent Kinase Inhibitor 2A

cDNA..... Complementary DNA

ENA78 ..... Epithelial-Derived Neutrophil-Activating Peptide 78

FU2 .....Follow up 2

FU3..... Follow up 3

FU4..... Follow up 4

GROα .....Growth-Regulated Oncogene-alpha

FTD.....Frontotemporal Dementia

GFAPα ..... Glial fibrillary Acidic Protein alpha

GM-CSF..... Granulocyte-Macrophage Colony-Stimulating Factor

GUSB..... Beta-Glucuronidase

HNRNP..... Heterogeneous Nuclear Ribonucleoproteins

HNRNPA0..... Heterogeneous Nuclear Ribonucleoprotein A0

HNRNPA1..... Heterogeneous Nuclear Ribonucleoprotein A1

HNRNPA2B1 ..... Heterogeneous Nuclear Ribonucleoprotein A2B1

HNRNPD..... Heterogeneous Nuclear Ribonucleoprotein D

HNRNPH3..... Heterogeneous Nuclear Ribonucleoprotein H3

HNRNPK ……Heterogeneous Nuclear Ribonucleoprotein K

HNRNPM…….. Heterogeneous Nuclear Ribonucleoprotein M

HNRNPUL2 …… Heterogeneous Nuclear Ribonucleoprotein U-Like 2

HPA …….. Human Primary Astrocytes

γIFN ……..Interferon gamma

IL-1B ……... Interleukin 1 beta

IL-2 ……. Interleukin 2

IL-6 ……Interleukin 6

IL-8 …….. Interleukin 8

IL-10…….. Interleukin 10

IMP3 …… IMP U3 Small Nucleolar Ribonucleoprotein 3

qRTPCR ……..Quantitative reverse transcription *Polymerase chain reaction*

LSM14A ……LSM14A MRNA Processing Body Assembly Factor

LSM2 …….. LSM2 Homolog, U6 Small Nuclear RNA and MRNA Degradation Associated

MMP ……..Matrix Metalloproteinases

MMSE ……..Mini-Mental State *Exam*

MMP3 …….. Matrix metalloproteinase 3

MMP10 …….. Matrix metalloproteinase 10

mRNA …….. messenger Ribonucleic acid

$p14^{ARF}$……..P14 alternate reading frame of the CDKN2A locus

PNISR……..*PNN Interacting Serine And Arginine Rich Protein*

PPIA……*Peptidylprolyl isomerase A*

RNA ……..Ribonucleic acid

SASP …… Senescence-Associated Secretory Phenotype

SF3B1…….. Splicing factor 3b subunit 1

SLC1A2……..EAAT2 glutamate transporter

SRSF1…….. Serine and Arginine Rich Splicing Factor 1

SRSF2…….. Serine and Arginine Rich Splicing Factor 2

SRSF3…….. Serine and Arginine Rich Splicing Factor 3

SRSF6…….. Serine and Arginine Rich Splicing Factor 6

SRSF7…….. Serine and Arginine Rich Splicing Factor 7

TIMP2……Tissue inhibitor of Metalloproteinases 2

TNFA.....*Tumor Necrosis Factor alpha*

TRA2B ……Transformer 2 Beta Homolog

1 INTRODUCTION

1.1.1 The burden of age-related neurodegeneration

Age is a major risk factor for cognitive decline and neurodegenerative disease, as it is for most common, chronic disorders (1). The process of biological ageing can be generalised as a gradual accumulation of molecular and cellular damage in an organism over time (2). The process is positively correlated with increased risk of chronic disease, and is the main risk factor for most common pathologies in the western world (3). Neurological ageing and associated neurodegenerative disorders (NDDs) characterise a group of disorders that have amongst the largest impact on health and quality of life for patients and their families; in 2015, 46.8 million people were living with dementia (4-6). These disorders also have profound economic consequences; the basic economic costs of dementia-related illnesses worldwide was estimated as 604 billion dollars in 2010 (7), with 70% of this cost focused in Western Europe and North America (4, 6). Dementia survival estimates vary from 1-8.5 years, depending on the age of onset; those being diagnosed earlier usually having increased survival time (8). Disorders characterised by cognitive decline include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS) and Multiple Sclerosis (MS)(9). Whilst specific disease processes may differ, generally the disorders disrupt motor, cognitive and sensory function, resulting in inability to perform physical and mental tasks, memory and emotional disturbance, and eventually death (9).

The pathology of NDDs is linked to loss or dysfunction of neurons, which progressively lose ability to react to stimuli and eventually degenerate (10). Inflammatory processes are linked to the pathology of these disorders (11). Protein aggregates such as β-amyloid or α-synuclein, along with neurofibrillary tangles consisting of Tau protein, present in brains affected by NDD's, have been reported to elicit

immune responses and potentially contribute to this inflammatory process (12). Ageing (3), infection (13) and trauma (14) have also been correlated to the process of neurodegeneration and associated pathologies.

Recent evidence suggests that the accumulation of senescent cells in the brain may actively drive functional decline in NDDs (15). The accumulation of senescent cells in tissues and organs has emerged as a major driver of the ageing process and age-related disease in mammals; selective removal of such cells in genetically-engineered animals has been demonstrated to reverse or delay aspects of ageing (16, 17). Senescence is a phenomenon whereby cells lose the ability to proliferate, and demonstrate functional decline and morphological differences to their non-senescent counterparts (18). Senescent cells are alive and metabolically active but have altered function compared to their non-senescent counterparts.

Although neurons are terminally differentiated and therefore non-proliferative, they are supported by replication competent cells such microglia and astrocytes. It is likely that senescence-related changes to the function of these cells may compromise their roles in metabolic and neuronal ion homeostasis (19) (20). Astrocytes have a pivotal role in maintenance of trophic factors, neurotransmitters, and the support and strengthening of neuronal connections (21). Previous evidence has suggested that the accumulation of senescent astrocytes may drive neurodegenerative disorders (22) and recent work shows that clearance of senescent glial cells may prevent the accumulation of Tau aggregates and improve cognitive function in mouse models (15, 23). Pharmacological impairment of astrocytic function recapitulates cognitive deficits that are observed in old age (24). It is also noteworthy that irradiation-induced accumulation of senescent cells is associated with cerebro-microvascular dysfunction in animal models (25).

A major feature of senescence is secretion of the senescence associated secretory phenotype (SASP), a cocktail of inflammatory cytokines which can act in a paracrine manner on neighbouring cells driving them into senescence or growth arrest. This catalyses a vicious cycle of senescence and inflammation in surrounding tissues and eventually throughout the entire organism, characterised in ageing (26) (27).This hypothesis is supported by the observation that senescent support cells exhibit a reduced capacity to support neurons grown in co-culture (28) and that analysis of the brains of patients with Alzheimer's associated dementia, demonstrate a significantly higher load of senescent cells and associated SASP factors (29).

Cells enter a senescent state for a number of reasons, one of which is repeated exposure to internal and external stressors (30). Exposure to cellular stress elicits an adaptive and plastic transcriptional response, which is partly orchestrated by alternative splicing (31). A large-scale gene enrichment study showed that successful or unsuccessful ageing (the primary risk factor for most high mortality pathologies), was most closely linked to perturbations in those genes which were linked to mRNA processing and splicing (32).

Supporting the notion of a pivotal role for dysregulated alternative splicing in senescence, transcripts associated with chronological age in humans are enriched in gene ontology pathways involved in splice site choice (32) and splicing factor expression is also associated with cellular senescence in *in-vitro* models (33, 34), with human ageing phenotypes (35) and with lifespan in long-lived mice (36). Perhaps most persuasively, restoration of splicing factor levels is associated with rescue of cellular senescence in multiple human primary human cell types (37-39).

Changes to the regulation of splicing have previously been reported in Alzheimer's Disease (40) and global dysregulation of splicing is characteristic of several neurodegenerative conditions such as Huntington's disease (41), frontotemporal lobar dementia (42) and Parkinson's Disease (43). Specially, alterations to the splicing pattern of the *MAPT* gene which encodes Tau protein is known to contribute to neurofibrillary tangles (44). Genes may be regulated at multiple levels, but recent studies have demonstrated the importance of mRNA processing in ageing and age-related disease (32, 45). Three types of mechanism primarily explain ageing related phenotypes: isoform based loss of function, aberrant gain of function and ratio imbalance (46).

1.2 ALTERNATIVE SPLICING

The stunning complexity of the gene regulatory mechanisms exhibited in human cells is being demystified at an ever-increasing rate thanks to rapidly developing technology, increased manpower and funding in the bioscience field (47). The central dogma script of molecular biology is being continually punctuated with newly-elucidated molecular control mechanisms and regulatory processes (48, 49), which add still more layers to the regulation of mRNA transcription, splicing, decay and translation, ultimately determining the flux in concentrations of various isoforms of mRNA transcripts which direct the molecular production of proteins (50). Over 95% of human genes are able to produce different isoforms through these this contributes in a pivotal way to the complexity of higher eukaryotes (51) (52).

Normal, constitutive splicing is the process of removing intronic sequences from pre-mRNA transcripts and binding together the resulting exon sequences to yield a functional, mature mRNA (53). This is achieved through number of co-transcriptional or post-transcriptional processes, of which pre-mRNA splicing is the most complicated (53).

Splicing occurs through a two-step, transesterification process occurring inside of a multi-megadalton ribonucleoprotein complex termed; the spliceosome. The spliceosome is the molecular machine through which much of the cellular splicing is done (54). It consists of 5 small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6) in addition to an impressive 150 associated proteins (55). A further central core of Sm proteins is needed for the assembly of the spliceosome, and methylation patterns of these directly affect their interactions and thus splicing (56). Assembly of the spliceosome is initiated first by the recognition of consensus sequences (most commonly GT/AG) at either end of the intron (57). U1 binds to the 5' splice site, two U2 subunits bind to the polypyrimidine tract (plays an important role in 3' splice site selection) and the 3' AG (58).

The mRNA transcripts are cleaved at the 5' splice site with the addition of a hydroxyl group to the splice site. The 5' end is then looped back around and ligated to the 3' branch site via a transesterification process, and the hydroxyl group is excised with the branched lariat loop (54) (see Figure 1.1).

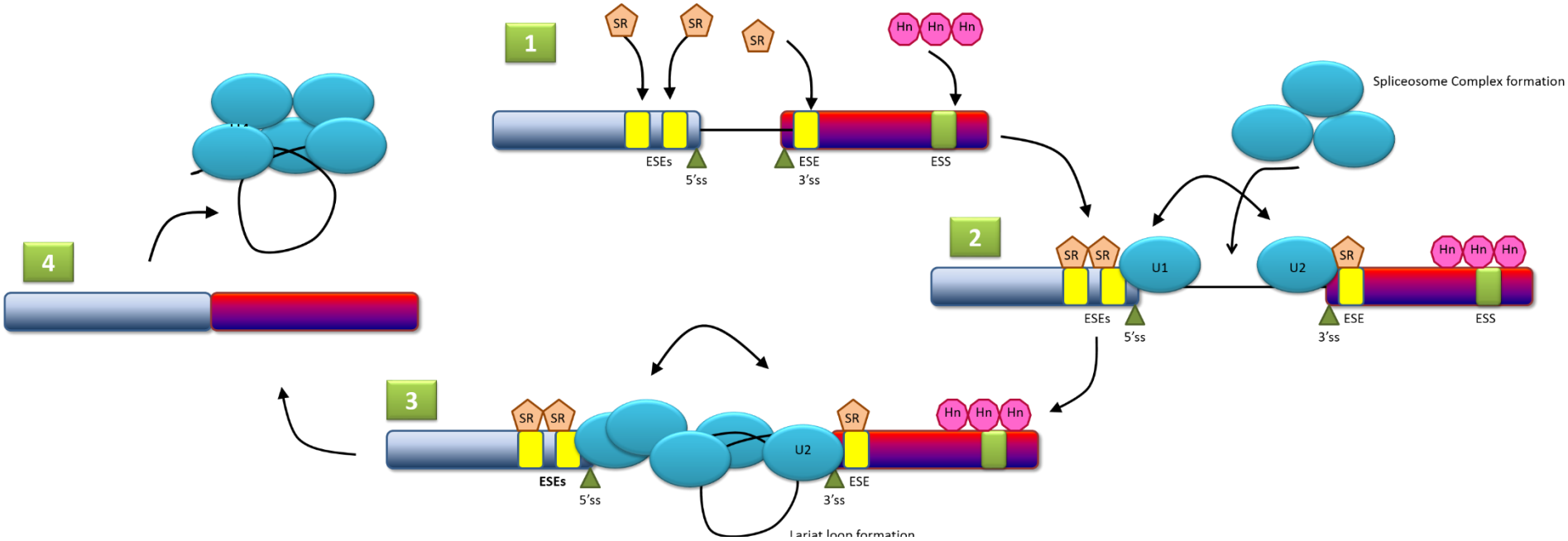

Figure 1.1 Constitutive and regulatory control of splicing processes (personal Communication - L.W.Harries)

1. Phosphorylated SR and hnRNP proteins bind to their respective intron/exon splicing enhancers and intron/exon splicing suppressors. 2. The U1 and U2 small nuclear ribonucleoproteins (snRNPs) bind to the 5' splice site (SD) and the branch point (BP) respectively. The tri-snRNP consisting of U4, U5 and U6 snRNPs then assembles. 3. A lariat loop is formed, by conformational change. And the U1 and the U4 snRNPs are displaced. 4. The intron is removed in the form of a lariat and 5. The exons are bound together by a transesterification reaction. SD = Splice donor site. SA = splice acceptor site. BP = branch point. SR = Serine Arginine rich splicing activator. HNRNP – Heterogeneous nuclear ribonucleoprotein particle splicing silencer. Exon/Intron Splicing Enhancer sites (ESE, ISE) are indicated by yellow boxes. Exon/Intron splicing silencer (ISS, ESS) binding sites are indicated by pale green boxes.

In addition, to this canonical molecular process, alternative splicing is the process of producing variations of these isoforms from a single pre-mRNA by including or removing exons from transcripts in a specific pattern (54). Alternative splicing is mediated by the combinatorial binding of a series of splicing activators and inhibitors to splicing enhancer and silencer sequences around the splice sites to determine whether or not each splice site is used (59, 60).In some instances exons are included which are normally excluded, in others an exon normally expressed can be spliced out for a mutually exclusive alternative (61). 4 basic alternative splicing patterns exist: Alternative 5' splice site selection, alternative 3' splice-site selection, cassette exon inclusion/ skipping, and intron retention (61). Mutually-exclusive alternative exons add an additional layer of complexity to these patterns (62) (See Fig 2). The resultant protein isoforms can have differential effects that range from mildly altered affinities, to demonstrating complete antagonism (63, 64).

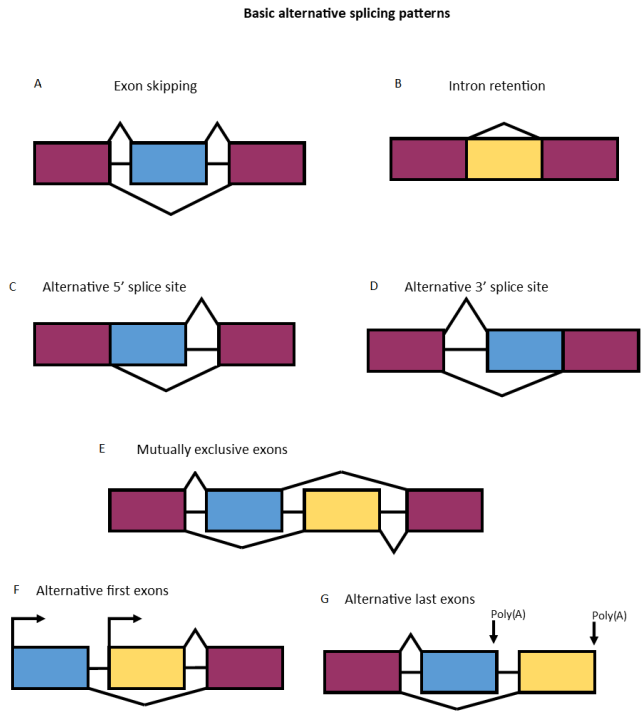

Figure 1.2- graphical representation of the patterns of alternative splicing.

Visual representation of the processes of splicing. Boxes represent exons joined by straight black lines representing the introns. Angular lines represent alternative splicing patterns and span the nucleic acid content to be removed. Examples refer to: A) exon skipping, B) intron retention, C) alternative 5' splice site, D) alternative 3' splice site, E) mutually exclusive exons, and alternative F) first and g) last exons.

Through this methodology, the genomic information present in a cell is able to generate an increased number of mRNA output variations and contribute to the increased tissue specific and temporal plasticity, facilitating adaptation to environmental pressures (65). The degree of transcriptional plasticity which can be achieved by this process is only now being fully unravelled. Computational genomic analysis of binding sites for splice factors revealed over 38,000 possible splice variants of a single gene (*Dscam*) in *Drosophila melanogaster* (66). Whilst this number isn't representative of the

vast majority of alleles, it goes some of the way to illustrating the vastness of the variation which exists beyond our basic genetic sequences. The resultant transcripts can have differential or antagonistic function, or in some cases splicing can produce non-viable transcripts by the introduction of premature termination codons and thus induction of the nonsense mediated decay (NMD) pathway (67).

The concentrations of, post-translational modifications to, and steric competition which occurs between *trans*-acting factors for cis-regulatory sequences for is a major driver of alternative splicing. Splice site usage (or not), is determined by the combinational balance of splicing inhibitors and activators (60). Splicing factors shuttle between nucleus and cytoplasm under the control of splicing regulator kinases that include Serine Threonine Protein Kinases 1 and 2 (SRPK1 and SRPK2) (68). Splicing factors are also themselves regulated by alternative splicing (67).

As transcription is controlled by the interaction between sequences in the DNA and proteins which bind to elicit initiation of transcription, so alternative splicing occurs when splicing regulatory activator or silencer proteins bind to exon or intron splicing enhancers (ESEs, ISEs) or silencers (ESS, ISS) in the pre-mRNA transcript (69). Sequence elements acting as enhancers or silencers are termed *cis*-regulatory sequencers, whilst those factors which bind to them are termed *trans*-acting factors (54). The relatively low affinity between splicing factors and mRNA is sometimes overcome by duplication of *cis* elements (70) . Splicing inhibitor proteins such as hnRNPs bind to exon splicing silencers, creating a loop out between the ESS bound molecule and the 5' splice site, thereby preventing the binding of SR proteins, and sequestering the exon from the pre-mRNA transcript (71). SR proteins, through their binding to enhancer elements bring about and stabilise a branch site (71). SR-like proteins are similar splicing effector proteins. They are not mandatory for the process of splicing, but have domains which are akin to those in SR proteins, enabling them to bind to splice sites in and can work antagonistically to the SR proteins (72). Some of these splicing factors which regulate splicing, are also regulated by

alternative splicing themselves (67), and a number are auto-regulated, whereby products bind to and control the splicing of their own transcripts, whilst also possessing the ability to bind to other transcripts with the same conserved sequence (73).

This network is then further complicated by the role which transcriptional control plays in initiation of the process, being as it is; co-transcriptional, as evidenced by chromatin immunoprecipitation (ChIP) and RNA polymerase knockout models (74) (75). Additional mechanism of alternative splicing control has been proposed based on results indicating endonucleases can induce splicing of the enzyme telomerase. Specifically the suggestion is that an mRNA transcript is produced from the complimentary strand, which is then digested forming a 47-mer oligonucleotide which binds at an intron exon junction – causing alternative splicing (76).

Finally, expression and function of splicing factors seems to be under the control of "master regulators" including Tra2β (77) Nova 1 (78), ERK and AKT which, through downstream kinase and phosphatase action, induce or prevent the binding of the transacting elements (79) . Investigation into the downstream targets of these pathways has yielded further potential points of intervention. Latorre *et al*. demonstrate that splicing factors can themselves be up-regulated, using the downstream targets or ERK and AKT, FOXO1 and ETV6 (80).

It stands to reason then, if the spliceosome is the most complex molecular machine in the cell, that 95% of the human genome is alternatively spliced, and master regulators control the elements of this splicing process, the potential ramifications of perturbations in the function or levels of these master regulators could be drastic.

1.3 DISRUPTED SPLICING IN THE AGEING BRAIN

1.3.1 The genetics of neurodegenerative disorders

Splicing disrupting mutations are those that through variants in the genetic sequence cause changes in the pattern of exon or intron usage (81). These are proposed to cause up to one third of all disease-causing mutations (82, 83). For the majority of cases however, there is not a single gene cause, with genetic variation in multiple loci and environmental factors being associated with these conditions (84).

Genome wide association studies (GWAS) are a powerful tool to identify genetic susceptibility factors for disease (85). GWAS studies have been employed to detect associations between common genetic variation and NDDs such as AD (86) ALS (87) PD(88) and for age of onset in HD (89). Reviews of all of the GWAS studies conducted since 2007, show a large number of associated loci, with all but one indicating APOEε4 confirmed as the most significant risk factor (90) which accounts for up to 50% of cases of late-onset AD (91). In 2013, meta-analysis of GWAS studies found 19 additional loci reaching genome wide significance ($P<5X10^{-8}$) additional loci for AD (92) and 24 additional loci for PD (93). GWAS studies also provide a foundation for investigation into treatment responses, allowing personalisation of therapeutic interventions in AD (94). Both monogenic (single gene) and multifactorial inheritance patterns for NDDs have been identified (95). Mutations in the following genes: Presenillin 1 (*PSEN1*) (96), α-synuclein (*SNCA*) (97), β-synuclein (*SNCB*), microtubule associated protein (*MAPT*) (98), Amyloid precursor protein (*APP*) (99) Huntingtin (*HTT*) (100), and Glial fibrillary acidic protein (*GFAP*) (101) are known to be monogenic causes of neurodegenerative diseases which demonstrate Mendelian patterns of inheritance within families.

1.3.2 Complex splicing patterns in the human brain

The brain shows some of the most complex patterns of alternative splicing (102). This is exemplified by the neurexin3 (*NRXN3*) gene. Neurexins are pre-synaptic extracellular scaffold proteins, and the balance of their numerous isoform types is imperative for normal synapse formation and transmission(103). *NRXN3* was originally believed to be capable of producing over 2000 different mRNA isoforms (104), although more recent studies suggest even this may have been an underestimate (103). The importance of the balance of the isoform ratio is a common theme throughout the transcriptome, both temporally and spatially (105, 106), and as such, control of isoform production is held under tight regulation (107).

Governance of alternative splicing through proteins such as Ptbp1 and Rbfox family members can control cellular differentiation fate in the development stages of the brain (108). Other examples include the Aquaporin 4 (*AQP4)* M1/M23 transcripts, the ratios of which ensure that osmosis occurs at a finely controlled rate to ensure homeostasis is maintained (109) (110). The Leucine rich repeat kinase 2 (*LRRK2)* gene has multiple isoforms produced by differential splicing of its 51 exons which because of different activities are preferentially transcribed in different tissues (111). Exons 5, is spliced out for the transcript occurring in astrocytes whereas the truncated exon 42a is produced in neurons and astrocytes, but is virtually undetectable in microglia (111). These are only a few examples of the importance of alternative splicing in the brain, the full complexity of which poorly understood.

1.3.3 Splicing dysregulation in neurodegenerative disorders

The potential for point mutations to disrupt the *cis*-acting regulatory elements of specific genes means that splicing disruption is common in disease (112). Splicing regulator genes have themselves been directly linked to NDDs (113). In addition, mutations in *trans*-acting elements, which bind to these will also cause disruption, but on a much larger scale, as these *trans*-factors are involved in the splicing of vast number of genes (114).

These *trans*-factor mutations cause metabolic and developmental problems (115) (114) and are sometimes lethal, as each splice factor will interact with cis-acting elements across a variety of genes (116). Similarly, if ageing or pathogenic processes disrupt the regulatory kinases and phosphatases directly linked to the activation and localisation of splicing factors, similar outcomes can occur as canonical splicing is dysregulated (117, 118). This is especially true of neurodegenerative disease (119) (120). Splice factors themselves are subject to further regulation through the action of phosphorylases and kinases. Phosphorylation of the SR and hnRNP proteins releases them from the cytoplasmic speckles in which they are normally localised (121), freeing them up to interact with the pre-RNA transcripts in the nucleus (122).

Examples of perturbation of such networks can be observed in existing cases of NDD's: alternative splicing of exon 10 of the *MAPT* mRNA, brings about two specific isoforms, termed 3R (inclusive of exon 10) and 4R (exclusive of exon 10) (123). Dysregulation of the *MAPT* splicing regulatory network and associated aggregation of pathological Tau, has been reported as a result of different phenomena. They include those caused by age-related and inflammatory mechanisms, aberrant activity of the regulatory kinases, and point mutations (120, 124). An example of pathogenicity resulting from phosphorylation status regulation in NDD's can be seen in the mechanism of Cdc2- like kinases (*CLK*) 1, 2, 3, and 4, which regulate exon 10 usage in Tau splicing through kinase action on SR proteins (125). Disruption of the exon 10 usage through CLK dysfunction produces imbalance between the Tau 3R

and 4R ratios, implicated in the development of AD (118). This is just one example, demonstrative of the ubiquitous presence and importance of alternative splicing throughout so many mechanisms and pathways in the cell, and how disruption at any stage can be catastrophic. Moreover, changes in transcriptional activity of essential splicing factors in peripheral whole blood is correlated phenotypes known to be associated with unsuccessful ageing such as decreased grip strength, and reduced performance on cognitive functions tests, believed to be mediated by the dysregulation of splicing (126).

1.3.4 Brain Inflammation and splicing regulation

The complex regulatory network between ageing, inflammation, splicing dysregulation and the development of NDDs is beginning to be unpicked, but there is still much to be done. Splicing factor activity is regulated by SRPK protein kinases and by FOXO1 and ETV6 via ERK/AKT signalling (127) (38, 128). Interestingly, initiation of inflammatory signalling of the SASP is brought about through NFκB pathway (129), which is also affected by the ERK and AKT signalling pathways (129). As a practical example, in mouse models of age related NDDs, increased transcription of inflammatory genes has been observed to correlate with subsequent dysregulation of the entire transcriptome and in addition dysregulation of alternative splicing and isoforms which affects synaptic plasticity (130).

Neuroinflammation is known to be linked to the development of NDDs, and can be brought about by a number of factors (131). GWAS studies have linked the innate immune system to sporadic AD development, indicating a link between neuroinflammation and AD related dementia pathophysiology (132). Review of the literature suggests inflammation drives neuronal damage, which triggers inflammation as part of a vicious cycle, and targeting neuroinflammation may provide an opportunity to treat AD (133). Similar findings are present in reviews of literature for ALS (134) and PD (135) in both of which microglia and astrocytes are activated in a similar manner to AD (128, 129). Splicing is also influenced by many factors which can include specific infectious agents such as Mycobacterium

tuberculosis (136). The adenovirus is another known pathogen which dysregulates splicing , which it achieves by taking over host cell splicing machinery, by dephosphorylating splicing factors; inhibiting endogenous splicing (137). Pathogens have been linked to the initiation of NDDs (13) which has been correlated with their severe dysregulation of alternative splicing (138) (139).

1.3.5 Brain stress response and splicing regulation

The brain is exquisitely sensitive to cellular stress. It is the most metabolically active organ in the body, and as such, has a high output of factors such as reactive oxygen species (140). Splicing regulators are also known to be very responsive to cellular stress (31) and represent a crucial part of the organism's repertoire of homeostatic mechanisms for dealing with changes in internal and external environment (141). Cholinergic hyper-activation brought about by physical or psychological stress in the CNS, brings about transcriptional activation and pre-mRNA splicing shifts (142, 143). In addition, changes in the RNA splicing of AChE transcripts has brain-to-blood effects, and can trigger neuronal – immune communication (144). Interestingly, suppression of these transcripts can reduce pro-inflammatory cytokine expression (144). Physiological stressors such as genotoxic agents, can induce changes in splicing patterns as a response to stress (145). For example, genotoxic stress can change the function of *MDM2* transcripts, promoting p53 activity in response to this stress (145). These stress-induced, splicing pattern changes can have a range of functions including producing non-productive variants to counter transcriptional upregulation, or alternatively the can induce apoptosis and senescence (145) (146).

1.3.6 Links between age-related changes and changes associated with neurodegenerative disorders

Age related changes which occur in the brain, often predispose to both neurodegeneration and to NDD's (147). These exist as both the accumulation of cellular and molecular damage, alongside breakdown in regulatory mechanisms (148, 149) and decline in the ability of the cells compensatory

mechanisms (147). As technology evolves allowing us to quantify the transcriptional changes common to ageing and to NDDs, interesting correlations begin to become apparent. Changes in alternative splicing patterns which are present in aged individuals are also present in those with a confirmed diagnosis of FTLD/AD, the difference in pattern is often only of magnitude (113). This may be due to age-related dysregulation of components of the core splicing machinery; activity levels of polypyrimidine tract binding protein (PTB) which aids in splice acceptor site recognition, was found to be consistent with these linked transcripts (113). Transcriptional changes directly linked to disorders, but not to age however, were consistent with decreased neuro-oncological ventral antigen (NOVA)–dependent splicing regulation (113).

The dysregulation of alternative splicing then, can come about by several mechanisms, and the subsequent effects in the brain are especially damaging. In an effort to unpick the problems, candidate genes known to be linked to the development of NDDs have been assessed for the production of multiple transcripts and for associated dysregulation of their concentration (150, 151). In AD, the delicate balance of isoforms with exon 10 inclusion/exclusion has been extensively explored and its dysregulation linked to the development of AD (152). In PD too, alternative splicing of a number of genes has been found to be linked to its pathogenic course (151).

Dysregulation of splicing is almost ubiquitous amongst NDD's. In AD, and Lewy Body Dementia, the presence of splice variants lacking the 7^{th} and 9^{th} exon of the glutamate transporter *EAAT2* appear to contribute to excitotoxicity, causing neurodegeneration (153), suggesting a common pathway of dysregulation. Mutations in the valosin-containing protein (*VCP*) gene was demonstrated to have a central role in development of ALS, mediated by premature intron retention in developmental stages (154) Glial fibrillary acidic protein, the primary intermediate filament in astrocytes, has alternative isoforms present in Alexanders disease (155), and an upregulation of normally low abundance

isoforms in AD – a phenomenon which has been tightly associated with increased plaque load(156). Some more specific examples are included below.

1.3.7 Alzheimer's Disease

Whole transcriptome analysis has provided evidence of large-scale changes across the transcriptome in Alzheimer's Disease (AD), with over twenty-seven thousand unique transcripts being reported in an AD brain (157). Genes involved in the pathogenesis of AD commonly have alternative splice variants, Amyloid Beta Precursor Protein Binding Family B Member 2 (*APBB2) , RNA binding fox homologue, (RBFOX), Presenilin1 (PSEN1), Presenilin 2 (PSEN2),* and Apolipoprotein E (*APOE*), have all been reported to have alternative transcripts which have been suggested to result in the aggregation of β-amyloid (150). Large scale splicing dysregulation has also been reported to create protein aggregates of many more types than previously believed. Using immunohistochemical analysis, core spliceosomal proteins such as the U1 snRNP were found to form aggregates themselves, implicating abnormal splicing more globally in AD pathogenesis (158). The brains normal splicing regulation appears to be particularly reliant on the splicing factor *NOVA,* and many of the dysregulated transcripts are targets of this protein (113). Decreases in levels of some neurotransmitters such as choline, which are associated with progressive AD, can also lead to decreases in the translation of splicing factor transcripts via the Nonsense-mediated decay (NMD) pathway (159).

Point mutations in splice donor sites in specific genes such as Amyloid precursor protein (*APP),* Presenilin1 *(PSEN1)* and Presenilin2 *(PSEN2)* genes have also been reported to produce aberrant transcripts that are also associated with β-amyloid aggregates (160-163). An intron inclusion transcript produced by an autosomal dominant *PSEN1* mutation has been causally linked to AD (162). The gene encoding the microtubule associated-protein Tau (*MAPT*), a major component of neurofibrillary tangles, also produces multiple transcripts, the balance of which has been heavily implicated in AD (123). Tau protein functionality is dependent upon its phosphorylation status, hyper-

phosphorylated Tau prevents its canonical role of binding to and stabilising microtubules (123). Mutations in the progranulin (*GRN*) gene, which encodes a pleiotropic anti-inflammatory protein, which has canonical neuronal protective effects, have also been linked to frontotemporal lobar degeneration (FTLD) (164-166). Aberrant splicing events around the first exon of *GRN,* leading to the introduction of premature termination codons, can lead to transcript degradation via the Nonsense-mediated decay (NMD) pathway with associated loss of neuroprotective benefits (167) (168). The *APOE* gene is one which has been thoroughly investigated after getting multiple GWAS hits but no splicing dysregulation has been reported for this gene in the brain of AD (169), however its receptor *APOER2* has been demonstrated to be dysregulated in both mouse and man (170) and restoration of correct splicing patterns was effective in preventing this mechanism of pathology (170).

1.3.8 Parkinson's disease

Whole transcriptome studies in PD have been mildly less successful in producing inter-study concordance; with no specific genes transcripts being ubiquitously de-regulated (171) (172). Grouping the significantly changed transcription levels into pathway groups however circumvents this problem (172).

α-synuclein (*SNCA*) gene has also been associated age at onset and disease progression in PD. *SNCA* produces several alternatively expressed isoforms, 4 of which are usually found only at low levels in unaffected brain. These isoforms have been reported to be overexpressed in different patterns in PD depending on the locality of the neurodegeneration (173-175). The same transcripts are also noted to be dysregulated in other NDDs, such as dementia with Lewy bodies and AD (176) (173, 177). It appears in some cases, that the presence of a known PD-associated SNP in the 3' region of the SNCA gene drives higher ratios of one of the transcripts which is present in large amounts in affected individuals (178) indicating that a *cis*-regulatory element may have been disrupted. Mutations affecting splice acceptor sites have been recorded in cis elements of presenilin-1 gene (179). Aggregation of misfolded

α-synuclein is a primary factor in pathogenesis of PD and dementia with Lewy bodies (DWLB) (180). Its oligomerisation leads eventually to the production of the intracellular aggregate known as Lewy Bodies (181). Alternative splicing of the α-synuclein transcript is one way in which misfolding can occur and lead to aggregation (182). The build-up of α-synuclein appears to be caused by transmission from the gut to the brain (183). Other genes with notable dysregulation of splicing in PD include leucine-rich repeat kinase 2 (*LRRK2*) implicated in neurite outgrowth, cytoskeletal integrity and autophagy. *LRRK2* boasts 51 exons harbouring mutations which elicit effects through disrupted alternative splicing. In addition, vacuolar protein sorting 35 (*VPS35*), parkin RBR E3 ubiquitin ligase (*PARK2*) (implicated in mitophagy metabolism cell growth and survival), and *PTEN* induce putative kinase 1 (*PINK1*).

1.4 SPLICING MODIFIERS AS FUTURE THERAPEUTICS FOR NEURODEGENERATIVE DISEASE

Whilst there are European Medical Association approved gene therapies now available for some genetic disorders such as GlyberaR® and Strimvelis®, much remains to be understood about the toxicity of these methodologies (184). Promising methods such as CRISPR/Cas9 technologies are still in very early stages, of investigation (184). Beyond gene therapy, three main points of intervention exist for tackling splicing. **1.** Co-transcriptionally targeting RNA with complimentary sequences to modify access by splicing components, or using RNAi to moderate levels of splicing factors, **2**) Modulation of how RNA targets are recognised by modification of the binding proteins, **3**) Modulation of the regulatory machinery which allow binding proteins to carry out splicing (185).

Modification of splicing patterns using targeted oligonucleotides or antisense technologies has enormous potential for exploration as future therapies (186). Small interfering-RNA based approaches have shown significant potential in cancer treatment when targeting the protein's kinases, which

directly affect the phosphorylation status and thus activity of splicing factors (187). The interactions between RNA binding proteins and RNA has been widely posited as a potential point for therapeutic intervention (113, 123, 188). However, the pharmaceuticals already in use with proven activity in the brain are non-selective (189), and so targeting specific interactions remains elusive. A more fruitful approach might lie in the identification of specific splicing changes with proven causality in driving neurodegenerative disease. Oligonucleotides which correct splicing dysregulation of the *APOER2* gene in the region of exon 19, demonstrate dramatic effects in mouse models of AD (190). Similarly, the Klotho protein involved in age suppression and longevity (191) has two commonly known isoforms, a secreted (s-Kl), and a membrane bound (m-Kl) form, which are produced by AS. Injections of the secreted form increases cognitive function, learning and memory in mouse models (192). A further form can be created by cleavage of the membrane binding domain of the protein, resulting in a different secreted form (193). Pharmacological intervention in the form of small molecules has also showed promise for rectifying dysregulation which accompanies ageing in other cells lines (37), and based on the congruencies between many NDDs and ageing in regards to RNA splicing this may hold promise for NDD interventions. Once compounds have been validated as modulating splicing in *in-vitro* studies, targeting them to the brain; a serious challenge – although one not without promising progress (194).

Finally, the development of therapeutics that specifically targeted the senescent support cells in the brain (astrocytes and microglia) might be an avenue for exploration. Senescence modulation has been suggested as a method for tackling the root causes of the age-related decline. These approaches involve clearance of senescent cells (senolytics) (195) or reversal of elements of the senescence program (senostatics) (196).

1.4.1 Summary

The hugely complicated process of alternative splicing is, because of this very nature of complexity potentially subject to so much error. In addition, as it is so ubiquitously involved with all the processes throughout the cell, when these errors occur, they will have far reaching consequences and in some cases be can be catastrophic. However, there is much promise as our technology and understanding advances further, and we can find enhanced methods of correcting the problems and re-balancing the delicate splicing process to combat disease and compress morbidity and delay mortality.

The links between NDDs and alternative splicing are clear and strong, and research into splicing modulators is still in its infancy. Research continues to identify causal links to, and potential therapeutic targets for, NDDs. Given the colossal importance of alternative splicing to the maintenance of healthy ageing, and the shared and intimate links to NDDs, targeting alternative splicing and its regulation promises to yield valuable information and potential therapeutic interventions. The advancement of gene therapy techniques that target specific isoforms or selectively target components of the splicing regulatory machinery may herald a real and valuable step forward towards the next generation of anti-degenerative therapies.

1.5 AIMS AND OBJECTIVES

Alternative splicing is of great importance to normal cell functioning and age-related perturbations are intimately linked to age related disorders, which in brain can manifest as neurogenerative conditions and associated cognitive decline. Cellular senescence is an age-related phenomenon, which appears to be mediated, in part, by alternative splicing. The accumulation of these senescent cells brings about chronic paracrine inflammation, driving further dysregulation of normal cell function, alternative splicing and evoking further cellular senescence in surrounding tissues.

Alternative splicing and age-related neurodegeneration – Jed Lye

We hypothesised that the phenomenon of cellular senescence in the astrocytes of the brain, contributes to the development of neurodegenerative disorders and associated cognitive decline, and that this was mediated by the senescence associated secretory phenotype, dysregulation of splicing mechanics and subsequent changes in isoform production.

We also considered, that if this was such, such isoform changes may be detectable in the blood, either through parallel changes in transcription organism wide, or through compromised integrity and breakdown in blood brain barrier, associated with age and NDDs. We aimed to create a senescent astrocyte population and characterise the associated secretory phenotype for this cell line. We then aimed to characterise splice factor dysregulation which occurs with cellular senescence and compare this with our characterised changes in isoforms of an *a-priori* gene panel in this cell line.

Once this had been achieved *in-vitro*, we aimed to conduct cohort study in which we compared peripheral whole blood levels of the isoforms we had identified, and using multivariate regression, asses the association between changes in these levels over a time course, and the development of cognitive decline.

2 METHODS

2.1 GENERAL METHODS

2.1.1 In-Vitro investigation into astrocyte splicing dysregulation

2.1.1.1 Cell culture

These studies used cultures of early-passage and late-passage human primary astrocytes (HPA) previously isolated from a block of sub-ventricular deep white matter tissue in a 17-year-old male donor immediately post-mortem with consent from next-of-kin. Ethical approval was granted by the North and East Devon Research Ethics Committee. Astrocytes were isolated from tissue blocks as previously described (197).

Human Primary astrocytes (HPA) were obtained from Celprogen (Celprogen Inc., Torrance, CA, USA) and at passage number 2. And cultured *in-vitro.* Cells were maintained in humidified incubators with 95% O_2/5% CO_2 in HPA stock media (Celprogen Inc., Torrance, CA, USA). Cell culture was performed in T75 culture flasks coated with poly-L-lysine (Sigma Aldrich, UK). To coat flasks, poly-L-lysine was diluted to 1:1000 in PBS, 2ml of this mixture was added to the flasks for 30 minutes before being discarded and flasks sealed with parafilm and refrigerated until use. Cells were seeded at a density of 400,000 cells per T75 flask. Cells were cultured in 10ml Celprogen human astrocyte cell culture medium with serum (M36058 -01s). Human astrocyte cells were cultured until 75-80% confluence was reached. Human astrocytes were washed twice with Dulbecco's PBS (Sigma Aldrich, UK), treated with 2ml 0.025% trypsin, neutralised with an equal amount of media, transferred to a flacon tube, pelleted using a centrifuge at 700xg resuspended in fresh media and counted. Counts were performed using the Biorad™, tc20 automated cell counter.

3 cryovials of human astrocyte culture was frozen down at each passage. Cell freezing was performed with a freezing mixture of 700ul human serum (Celprogen Inc., Torrance, CA, USA) 200ul cells in medium and 100ul DMSO (Sigma Aldrich, UK). These were then placed in 'Mr. Frosty' tool, to reduce the rate of freezing and reduce crystal formation and placed in -80^{0}C freezer.

For the production of senescent cultures, cells were counted, and equal numbers of cells seeded (4x10^3 cell/cm^2) at each passage in continuous culture until the growth of the culture slowed to less than 0.5 population doublings (PD)/week. Astrocyte cultures underwent continuous culture until the onset of replicative senescence and growth arrest in 3 biological replicates. Early passage astrocytes at PD = 24 and late passage astrocytes at PD = 84 were used.

2.1.2 Quantification of senescent cell load

To quantify senescence in early and late passage astrocytes, 3 biological replicates were evaluated for activity of the biochemical senescence marker senescence-associated β galactosidase (SA β-Gal) using a commercial kit (Sigma Aldrich, UK) according to manufacturer's instructions, with a minimum of 100 cells assessed per replicate.

Briefly, cells were seeded into 6 well plates which had been ready -coated with Poly-L-lysine at a density of 60,000 cells per well and incubated in 2ml astrocyte media from Celprogen at 37^{0}C until these reached 75% confluence.

At this point, media was aspirated off, cells were washed twice, and 1.5ml fixing solution was applied. Cells were then incubated for 5 minutes. Fixative was pipetted off, and cells were washed three times more with PBS, and finally 1.5 ml of staining solution (see Table 2.1) was added to each well. We noticed that if we followed the manufacturers guidelines and added a 2ml volume of the staining

solution to each well, the amount of blue stain produced by the cleavage reaction was too great and it was not possible to differentiate between those cells which were stained and those which were not. Amounts were reduced by 25%.

Table 2.1 Staining solution reagents

Reagent	Measure
X-gal solution (pre-warmed)	0.25ml
Reagent A	125ul
Reagent B	125ul
DDH2O	8.5ml

Importantly the staining reaction is a redox reaction and particularly sensitive to changes in Ph. Manufacturer's instructions including sealing the plate with parafilm and being sealed in a non-co2 incubator. Cells were then examined through microscopy and counts of stained cells were obtained and compared between young and old astrocytes to determine the overall levels of cellular senescence between samples.

Senescence was also quantified by assessing the expression of the *CDKN2A* gene (a known molecular marker of cell senescence) and by changes in cell morphology typical of senescence as in our previous work (33, 37). Total RNA (100ng) was reverse transcribed in 20 µl reactions using EvoScript reverse transcriptase (Roche Life Sciences, Burgess Hill, UK). Total *CDKN2A* expression was measured by qRT-PCR relative to 3 empirically-determined endogenous control genes (*GUSB*, *PPIA* and *GADPH*) on the

QuantStudio 12K Flex platform (Applied Biosystems, Foster City, USA). PCR reactions contained 2.5 µl TaqMan Universal Mastermix (no AMPerase) (Applied Biosystems, Foster City, USA), 900nM of each primer, 250nM probe and 0.5 µl cDNA in a total volume of 5 µl. Cycling conditions were a single cycle of 95 ºC for 10 minutes followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute.

2.1.2.1 Profiling of Senescence associated secretory phenotype in young and old cells

Early and late passage human astrocytes were seeded in two biological replicates of $10x10^4$ cells in a $25cm^2$ flasks pre-lined with Poly-l-Lysine as described above at a density of 100,000 cells. After 48hours incubation, cell supernatants were harvested and stored at -80°C. SASP was profiled using both ABCAM Human Cytokine Antibody Array (ab133997; Abcam, Cambridge, UK) and the ABCAM Human MMP Antibody Array (ab134004; Abcam, Cambridge, UK). SASP components measured were: IL-1B, IL-2, Il-6, IL-8, IL-10, TNFα, IFNγ, GM-CSF, Angiogenin, ENA78, GROα, MMP3, MMP10 and TIMP2.

Cell membranes from the kits were blocked with supplied blocking buffer at room temperature for 30 mins, before aspirating off blocking buffer. 1ml of media was then applied to each membrane and incubated for overnight at room temperature. Following overnight incubation, membranes were then washed for 30 minutes, in 20 ml of supplied wash buffer 1. Six more subsequent washes of the membranes were then conducted, 3 each with both wash buffer 1, then wash buffer 2. Biotin-conjugated anti-cytokines were then pipetted into each well and incubated for 2 hours, at room temperature. The anti-cytokines were then aspirated off, and the 6 washes with wash buffer 1 and 2 were repeated. 2ml of HRP-conjugated streptavidin was then applied into each well, and the membranes were incubated for 2 hours at room temperature. 6 washes were then repeated once more, 3 each with wash buffer 1 and 2.

Membranes were transferred to tissue paper to dry. Excess buffer was removed by blotting the edges of the membrane with tissues paper, while preventing the membranes from drying out completely. Membranes were transferred to provided plastic sheet. 500µl of pre-mixed detection buffer C and detection buffer D were pipetted onto the membrane, taking care not to disrupt the reagents already bound to the membrane, and incubated for two minutes. A final plastic sheet was then applied to the top of the membrane surface, and membranes were imaged using Bio-Rad chemi-doc chemiluminescence. Images were interpreted using image J software, and the absorbance of each cytokine and MMP antibody was compared between the samples of media taken from old and young astrocyte cultures. Results were normalised using positive and negative controls as per the kit instructions using the LI-COR Odyssey® CLx imaging system (Lincoln, NE, USA). An unpaired two tailed t-test was used to assess statistical significance in secreted matrix metalloproteinases and inflammatory cytokine secretion using Minitab 18 software package (Minitab, Centre County, USA).

2.1.2.2 RNA extractions

Cells were treated with 1ml trypsin for 1 minute, to detach from poly-L-lysine. Cells aspirated from the flasks and added to an Eppendorf tube. Trizol was added to lyse cells, and falcon was inverted 5 x and then incubated at room temperature for 5 minutes. 0.2 ml of chloroform was added, and tubes were shaken for 10 seconds and incubated at room temperature for a further 3 minutes. Falcons were then centrifuged at 12000 rpm for 15 minutes at 4^{o}c. The aqueous phase is was then transferred to a fresh Eppendorf. 0.5ml of isopropanol was then added to the solution, to facilitate the precipitation of the RNA, and this was left overnight. 24 hours later, samples are centrifuged at 12000 rpm for 10 minutes to form a pellet. Supernatant was then pipetted off, and 1ml of 75% ethanol was added to wash the pellet. RNA was then pelleted again by centrifugation at 1200 x G for a further 30 minutes, this is then resuspended in 500ul 75% ethanol. This Eppendorf is then left open, for the ethanol to evaporate, and finally the RNA is then diluted in 10ul RNase free water.

2.1.2.3 Reverse Transcriptions

Reverse transcriptions were carried out using The SuperScript VILO cDNA Synthesis Kit, (Thermo Fisher Scientific, Warrington, UK). All regents were mixed on ice. Primarily, 2µl of 10x superscript enzyme mix was combined with 5x VILO reaction mix, 2ul RNA (with total amount of RNA being 100ng), and 11 ul of DDH2O. Mix was then incubated at 25^{o}C, for 10 minutes (annealing step), followed by 42 ^{o}C for 60 minutes (extension step), and finally 85 ^{o}C for 5 minutes (denaturation step).

2.1.3 Quantification of splicing factor expression

Splicing factors have previously been demonstrated to be associated with cellular senescence with evidence suggesting they may be drivers of this process in some tissues (37-39). We measured the expression levels of an a priori panel of 20 splicing factors previously associated with age, lifespan, cellular senescence in different tissue types in our previous work (32-34). This panel included the splicing inhibitors *HNRNPA0, HNRNPA1, HNRNPA2B1, HNRNPD, HNRNPH3, HNRNPK, HNRNPM, HNRNPUL2,* the splicing activators *AKAP17A, PNISR, SRSF1, SRSF2, SRSF3, SRSF6, TRA2B*, *SRSF7* and the *SF3B1, IMP3, LSM14A,* and *LSM2* components of the core spliceosome. Splicing factor expression was measured in 3 biological and 2 technical replicates by qRT-PCR using custom TaqMan Low Density Arrays (TLDA) on the Quantstudio 12K Flex platform as previously described (33). Transcript levels were expressed relative to the geometric mean of the *GUSB* and *PPIA* endogenous control genes and normalised to their expression in RNA from early passage cells. We then performed tests for equality of variance and t-test using IBM SPSS Statistics 25.

2.1.4 Quantification of candidate gene expression in early passage and senescent astrocytes

A panel of candidate genes were selected for analysis on the basis of biological relevance; known links with brain function, neurodegenerative disease or senescence, and where available, evidence from the literature that alternatively expressed isoforms may have differential function to allow interpretation of changes. The identity of genes tested, and brief description of function is given in Table 2.2.

TaqMan Assays specific to isoforms were designed to unique regions of the transcripts in question (assay sequences are available upon request). Assays were validated by standard curve analysis using 7 serial 1:2 dilutions of cDNA derived from whole brain lysate. Reverse transcription and qRT-PCR conditions are described above. Experiments were carried out in 3 biological and 3 technical replicates. Again, transcript levels were expressed relative to *GUSB* and *PPIA* endogenous control genes and normalised to their expression in RNA from early passage cells. We then performed tests for equality of variance and t-test using IBM SPSS Statistics 25.

Table 2.2– Transcript isoforms identified for expression analysis.

The table gives the identity of the isoforms selected for analysis, the relevant NM accession numbers and a brief description of their function.

Gene	**Transcript Accession**	**Isoform/transcript function**
ATM	NM_000051.3	DNA Damage repair
AQP4		
AQP4M1	NM_001650.6	Pore-forming Integral membrane protein

AQP4M23	NM_004028.4	Pore-forming Integral membrane protein
SLC1A2		
EAAT2A	NM_004171.4	Excitatory amino acid transporter
EAAT2B	NM_001252652.1	Excitatory amino acid transporter
GFAP		
GFAPA	NM_002055	Astrocyte intermediate filament protein
GFAP(B)	NM_001131019.1	Astrocyte intermediate filament protein
KL		
KLOTHO mKl	NM_004795	Membrane bound isoform, coreceptor for FGF23
KLOTHO msKl	NM_004795.3	Secreted isoform, endocrine factor which improves cognitive performance in ageing
CDKN2A		
p14^{ARF}	NM_058195	p53 pathway to cell cycle cessation
p16^{INK4A}	NM_001195132	RB1 pathway to cell cycle cessation (198)
CDKN1A		

p21a	NM_078467	Inhibits proliferation
p21b	NM_000389	Promotes proliferation
TP53	NM_001126118, NM_000546, NM_001126112, NM_001276696, NM_001126113, NM_001276699, NM_001276698, NM_001276697	Cell cycle regulation
PSEN2	NM_012486.2	Processing of β-amyloid
MAPT		
TAU3	NM_001203251.1 NM_001203252.1 NM_016841.4	Microtubule protein involved in neurofibrillary tangles
TAU4	NM_001123066.3 NM_001123067.3 NM_005910.5 NM_016834.4	Microtubule protein involved in neurofibrillary tangles

	NM_016835.4	

Probes were designed by obtaining the genomic sequence of the transcripts using the UCSC Genome browser database and performing *in-silico* splicing of the sequence and designing probes bridging specific exons boundaries which only existed in the target isoforms, these were ordered from Thermofisher™. In the case that Thermofisher™ had off-the-shelf probes ready designed for an isoform species, these were ordered and efficiency of 100% was assumed; therefore these probes have values which appear blank in the table. Probe binding efficiency was validated via QPCR standard curve in whole blood (Table 2.3) Differential expression of the candidate isoforms in young and old primary astrocyte cell lines were measured using qRT-PCR, and expression changes were calculated using the $\Delta\Delta$CT method, which is explained below.

Table 2.3 Standard curve binding efficiency assay

All probes had binding efficiency over the pre-designated threshold of 80% except for *P2SV* which did not successfully bind and was subsequently removed from the study. Gradients lower than -3.32 indicate less than 100% efficiency. R^2 values are indicative of pipetting error, with a value of 1 being ideal. The values were deemed to be acceptable.

Gene	Slope	y	R^2	Binding efficiency
p53				100
ATM				100
GADPH				100
Gfapa	-3.563	30.347	0.999	90.823
gusb				100
mKlotho	-3.882	38.348	0.947	80.959
p14				100
P16				100
p21b				100

p53				100
ppia				100
PSEN2	-3.663	34.477	0.995	87.49
P2SV	0	0	0	0
sKLOTHO	-3.589	37.363	0.983	89.956
tau3	-3.792	35.832	0.991	83.53
tau4	-3.8	31.594	0.94	83.307

2.1.4.1 ΔΔCt method

We trialled 3 common housekeeping genes *PP1A*, *GUSB* and *GAPDH* for comparison in expression changes across the candidate gene list. Once qRTPCR had been conducted we used the online tool ref-finder (decommissioned) to ascertain which method of normalisation would be optimal based on the stability of expression. Methods trialled were each of the housekeeping genes (HKG's) individually, combinations of two HKG's, all three HKG's, geomean of all samples, geomean of all samples plus HKG's Geomean of all samples, plus combinations of two HKG's and geomean of all samples plus individual HKG's. It was determined that the most stable baseline for control was the geomean of all samples, plus *PP1A* and *GUSB*. *GAPDH* was observed to be very unstable in our astrocyte samples and was removed from all analysis. When calculating the differences between the cycle threshold (CT) of baseline expression and the expression of each sample, we used the median values of the triplicates to mitigate the effect of any outliers. The difference (Δ) between CT of baseline and RNA isoform was compared for both young and old astrocytes (ΔΔCt), and log base 2 was taken of these values to derive fold change, this is the basis for the semi- quantitative 2ΔΔCt method which is used for deriving the relative abundance of gene or isoform expression.

2.1.5 Assessment of candidate gene expression levels with cognitive decline in a longitudinal human population

The temporal study of cognitive decline and associated factors requires a study length beyond that of the allotted time of the current study. We have therefore made use of the epidemiological prospective population-based "InCHIANTI" study. Inverchiare in Chianti (ageing in the Chianti area) is an in-depth cohort study, providing the data from people of age range 20-102 years living in the Chianti geographic area (Tuscany, Italy) with detailed assessment of health and lifestyle parameters at baseline, and again at 4 subsequent follow-ups (FU2; 2004 – 2006, FU3; 2007 – 2009 and FU4; 2012 - 2014) (199).

The study's principal investigator Dr. Luigi Ferrucci initiated the study in 1998, supported by a grant from the National Institute on Ageing. The study was designed to enhance comprehension of the risk factors for age related disorders later in life, and to produce a standardised schematic for metric detailing and observation relating to ageing and associated pathologies. Physiological factors, which affect age related pathology, were broken down into 6 subsystems: the central nervous system, the peripheral nervous system, the perceptual system, muscles, bone-joints, and energy production/delivery. Basal data collection started in 1998 and was completed in 2000, with follow-ups of each participant every 3 years subsequently. These time-point intersections at which data was collected and clinical examinations were conducted were termed "waves". The examination included vast amounts of survey questions about lifestyle, diet, education, background, physical health, and involved acquisition of samples of blood for analysis.

For the initial study, samples were obtained from two specific areas – one urban (Greve in Chianti) with a population of 11,709 inhabitants of which 19.3% are 65+, and one rural (Bango a Ripoli) with 4704 inhabitants of which 20.3% were 65+. A two-stage sampling procedure was used at both to obtain representative samples. Initially, 650 persons over the age of 65 were selected from the population registry, with the caveat of Italian birth being a pre-requisite for study inclusion. In

addition, 50 men and 50 women were then selected randomly from each 10-year age group from 20 to 59, with 10 men and women from the age group 60-64.

We selected 197 participants from this cohort for study. Inclusion criteria were age at FU3 > 64 years with an MMSE score > 18 to avoid those already on a declining cognitive trajectory and availability of an FU3 RNA sample with clinical information available at both FU3 and FU4. Participants were categorized into 'mild' or 'severe' groups depending their change in the MMSE score between FU3 and FU4; individuals declining between 2 and 8 points were defined as 'mild' whereas those declining between 9 and 22 points were categorised as 'severe'. These thresholds were chosen on the basis of previously defined criteria where a 'severe' decline was categorised as a drop in MMSE score > 3 points per annum (200-202). Ethical approval was granted by the Instituto Nazionale Riposo e Cura Anziani institutional review board in Italy. Methods were carried out in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants.

2.1.6 Blood Collection

Peripheral Blood samples were collected from 700 of the participants within 3 weeks of the 9-year follow up interview. Participants were instructed to fast for at least 8hrs prior to collection. 2.5ML peripheral whole blood was collected into PAXgene tubes (BD Biosciences) at wave 9 and wave 12 and frozen at -80^{o}C and RNA was extracted using the PAXgene blood RNA kit (Qiagen, Paisley, UK) as per manufacturer's instructions.

RNA extractions were performed using the Pre-analytix whole blood RNA extraction kit on the whole blood of samples obtained from individuals from the InChianti cohort at the two separate time points (wave 9 and wave 12). Reverse transcriptions were carried out on 100ng RNA using the EvoScript™ Universal cDNA Master kit (Roche Life Sciences, Burgess Hill, UK) according to the manufacturer's instructions except for a change to the extension phase of the reaction: a step of 30 minutes at 65°C

was used instead of 15 minutes at 65°C. reactions were set up in a nuclease free environment, and placed on ice. Reagents were added to the Eppendorf in according to manufacturer's instructions (see Table 2.4). The enzyme mix was added last, before which all reagents were vortexed.

Table 2.4 RTPCR reaction mix

Reagent	Volume
Water	Make up to 18ul
Reaction buffer	4ul
Template RNA	100ng
Enzyme Mix	2ul

RtPCR thermocycler was set up as follows:

- 42°C for 15 minutes.
- 85°C for 5 minutes.
- 65°C for 30 minutes.
- 4°C with an unlimited Hold time.

Concentration of DNA was assessed using the Thermo Scientific NanoDrop 2000. We then assessed expression of transcripts which had observed senescence-related dysregulation in levels in-vitro in peripheral blood. These were GFAPa, Mklotho, Tau3, PSEN2, P14, P16, P21a and P21b. Relative

expression of mRNA isoforms was measured through qRTPCR. This was achieved using 96well plates on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System. Samples were run in triplicate; batch effect was corrected for by cross plate normalisation to control samples.

We then investigated the association between transcript expression and cognitive decline as measured by change (Δ) in mini mental state exam (MMSE) score. The MMSE was developed by Folstein and Folstein in 1975 as a method to assign quantifiable metrics to the intellectual abilities and deterioration thereof in patients. It has become the most common clinically utilised test of cognitive function (203). The MMSE takes around 10 minutes to complete, consists of both verbal and written participation and measure orientation, registration, attention calculation recall and language (204). It also has a higher sensitivity than other commonly used assessments (205).

To conduct our analysis we organised the samples into two groups, those with a mild cognitive decline; as evidenced by a decrease in MMSE score of between 2 and 8 points over a 3-7 year period, or those with severe decline which was represented as a reduction of 9-22 points during that same period.

A mild reduction was classified as a reduction of 2-8 points in MMSE performance over a 3-7-year period or severe decline. Severe reduction classification was a reduction of 9-22 points in MMSE performance over the same period. Using multivariate linear regression models with age, gender, smoking (lifetime pack-years), study-site, education level and white blood cell subtype counts (% neutrophils, monocytes, basophils, eosinophils) entered as confounding factors.

3 RESULTS

Introduction

We hypothesised that senescent astrocytes would display differential expression of splicing regulatory factors and altered patterns of alternative splicing *in vitro*, and that some of these isoform changes may be detectable in peripheral blood and show statistical associations with cognitive phenotypes in human populations.

We first characterized the astrocyte SASP in terms of cytokine and MMP production, and then determined the splicing factor repertoire and patterns of alternative splicing for a panel of brain or senescence candidate genes in senescent human primary astrocytes. Where dysregulation of splicing patterns was demonstrated and expression was conserved in blood, we then assessed associations between peripheral blood isoform levels and measures of cognitive dysfunction from 197 individuals from the InCHIANTI study of aging, a longitudinal and cross-sectional population study of individuals from the Tuscany region of Italy (199). We identified that senescent astrocytes display a modified SASP, consisting of elevated IL8, MMP, MMP10 and TIMP2 levels, but decreased IL10. 50% of splicing regulatory factors tested demonstrated dysregulated expression in senescent astrocytes; this was accompanied by altered splicing of 7/13 of candidate genes tested. Furthermore, when we assessed the relationship between peripheral blood expression of isoforms dysregulated in astrocytes and cognitive decline as measured my Mini Mental State Exam (MMSE), *GFAPα* and *TAU3* transcript levels were positively correlated with cognitive decline, whereas GFAPA transcript levels were negatively-associated with cognitive decline over a 3-7 year period in participants from the InCHIANTI study of aging. Our data agree with the hypothesis that senescent astrocytes display differential expression of splicing regulatory factors and altered patterns of alternative splicing, and that some of these isoform changes may reflect those in peripheral blood. Such changes may show statistical associations with cognitive phenotypes in human populations.

3.1 RESULTS

3.1.1 Characterization of senescent astrocytes

Human primary astrocyte cultures were passaged until PD84 at which point they were considered senescent. This was verified by molecular and biochemical characterisation of the growth kinetics of the cultures; senescence associated β-galactosidase (SA-β-gal) staining demonstrated a significant increase in the number of senescent cells from 8% in early passage cells to 36% in late passage cells (Figure 3.1 A) which was mirrored by a concurrent increase in the expression of the *CDKN2A* gene Figure 3.1 B). SASP factors in conditioned media derived from senescent cells demonstrated altered levels for several key SASP proteins; we observed elevated IL-8, GM-CSF, Angiogenin, ENA78, GRO-α, MMP-3, MMP-10 and TIMP2 levels (Table 3.1, Table 3.2, Figure 3.2, Figure 3.3). Several other changes in SASP profile were also seen to be approaching statistical significance; IL-12, MCP-2 and MIP-1o all saw increases in secretion at levels approaching P=0.05.

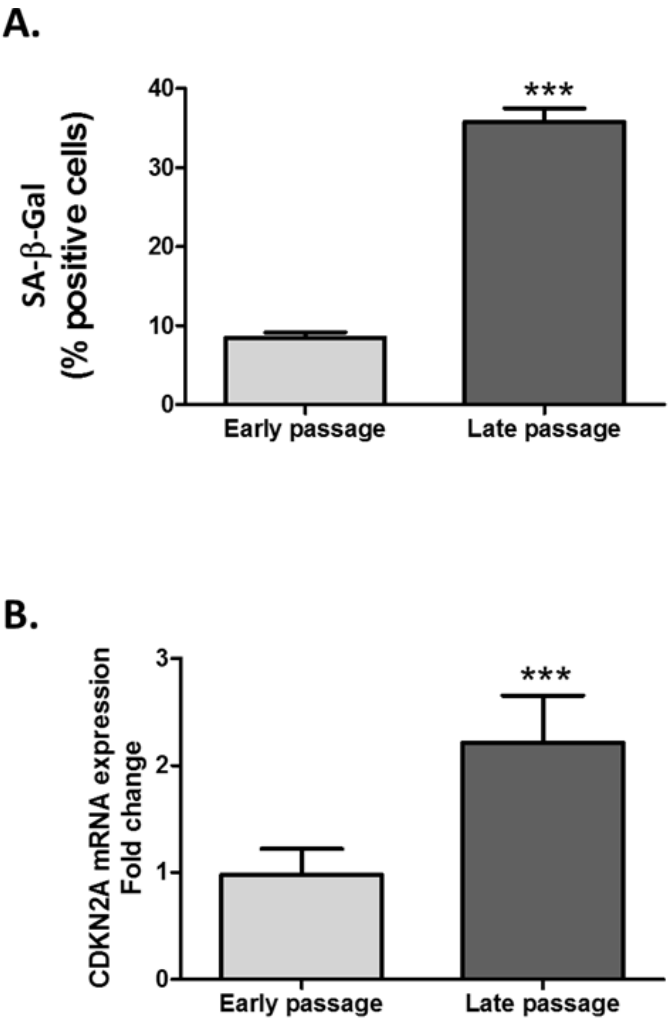

Figure 3.1 Senescence validation

Change in senescent cell load as determined by senescence-associated β-galactosidase (SA-β-Gal) staining between early passage and late passage human primary astrocytes. The percentage of cells staining positive for SA-β-Gal is given on the Y axis, and the identity of the cell culture on the X axis. Early passage cells are population doubling (PD) 24, late passage cells are at PD = 84. Results are from 3 biological replicates. * = p<0.01, **=p<0.01, ***=P<0.001. Error bars refer to Standard deviation of measurement.

Table 3.1 Profiling the senescence associated secretory phenotype in astrocytes - Cytokines

Table shows Gene ID, mean cytokine secretion *in-vitro* for young (Y) and old (O) astrocytes in arbitrary units, standard deviation, log-base 2 of fold change (n=6). T values and P values. Statistically significant (P <0.05) changes are denoted by * and row is shown in bold typeface, highlighted grey. Those approaching but not meeting statistical significance are shown in in bold typeface.

Gene	Mean Y	SD	Mean O	SD	Log 2FC	T	p
ENA78	**160.6179**	**54.7**	**270.2554**	**54.7**	**0.75**	**-2.76**	**0.033***
GM-CSF	**27.05994**	**7.63**	**66.12956**	**15.3**	**1.29**	**-4.58**	**0.04***
GRO	442.2389	134	528.5193	94.7	0.26	-1.05	0.334
GRO-a	**256.1518**	**55.6**	**461.5086**	**130.2**	**0.85**	**-2.902**	**0.027***
I-309	56.35094	17.0	53.72426	32.7	-0.07	0.125	0.905
IL-1a	79.76639	25.7	76.30872	17.3	-0.06	0.224	0.831
IL-1b	274.2334	48.7	286.058	73.6	0.06	0.27	0.798
IL-2	74.65903	48.0	111.5412	37.9	0.58	1.21	0.273
IL-3	258.89	58.7	293.8777	58.7	0.18	0.98	0.363
IL-4	**99.51305**	**11.2**	**120.0094**	**17.0**	**0.278**	**2.01**	**0.091**
IL-5	91.63144	4.1	63.09812	37.6	0.54	1.508	0.182
IL-6	78.75196	23.6	73.4896	19.0	-0.10	3.47	0.740
IL-7	84.55205	27.3	84.66937	29.0	0.00	-0.06	0.995
IL-8	**409.5415**	**111.9**	**1097.73**	**256.3**	**1.42**	**-4.921**	**0.003****
IL-10	56.5598	19.1	18.21913	9.6	-1.63	3.595	0.11
IL-12p40/P70	**144.4992**	**32.2**	**213.145**	**55.9**	**0.56**	**-2.131**	**0.077**
IL-15	148.8075	40.6	186.7788	43.8	0.33	-1.272	0.251
IFN-y	120.7869	40.1	137.9333	47.8	0.19	-.550	0.602
MCP-1	13789.02	2275.5	14939.13	4300.9	0.12	-.473	0.653

MCP-2	**243.1771**	**53.8**	**325.7858**	**51.0**	**0.42**	**-2.230**	**0.067**
MCP-3	84.43644	26.2	108.8109	34.6	0.37	-1.122	0.305
MCSF	329.5096	59.6	374.0884	57.7	0.18	-1.075	0.324
MDC	313.7771	42.9	339.8177	97.7	0.12	-.488	0.643
MIG	45.36578	25.8	55.28631	49.0	0.29	-.358	0.732
MIP-1o	**172.7754**	**30.9**	**235.2977**	**53.1**	**0.45**	**-2.035**	**0.088**
RANTES	483.5395	108.8	573.9288	150.5	0.25	-.973	0.368
SCF	250.5389	56.4	293.2824	95.3	0.23	-.772	0.469
SDF-1	467.7116	140.7	394.8161	153.0	0.24	.702	0.509
TART	325.1283	44.7	399.2366	96.1	0.30	-1.398	0.211
TGF-b1	177.1489	25.1	253.0473	103.0	0.51	-1.432	0.202
TNF-a	10.21346	11.3	16.81171	16.2	0.72	-.598	0.576
TNG-b	219.506	131.6	126.9624	19.4	0.79	1.392	0.213
EGF	144.5994	32.3	132.2963	75.2	0.13	.301	0.774
IGF-1	131.1638	50.4	167.6447	36.5	0.35	-1.173	0.258
Angiogenin	881.4542	213.9	1397.67	206.7	0.67	-3.471	0.13
Oncostatin M	1157.461	256.7	950.4137	129.2	0.28	1.441-	0.200
Thrombopoietin	249.2383	69.8	312.3784	62.4	0.33	1.349	0.226
VEGF	376.0153	92.0	483.9697	89.2	0.36	-1.685	0.143
PDGF BB	230.6906	73.9	231.5844	35.6	0.01	-.022	0.984
Leptine	178.1742	32.99756	234.2760	72.7	0.39	-1.406	0.229

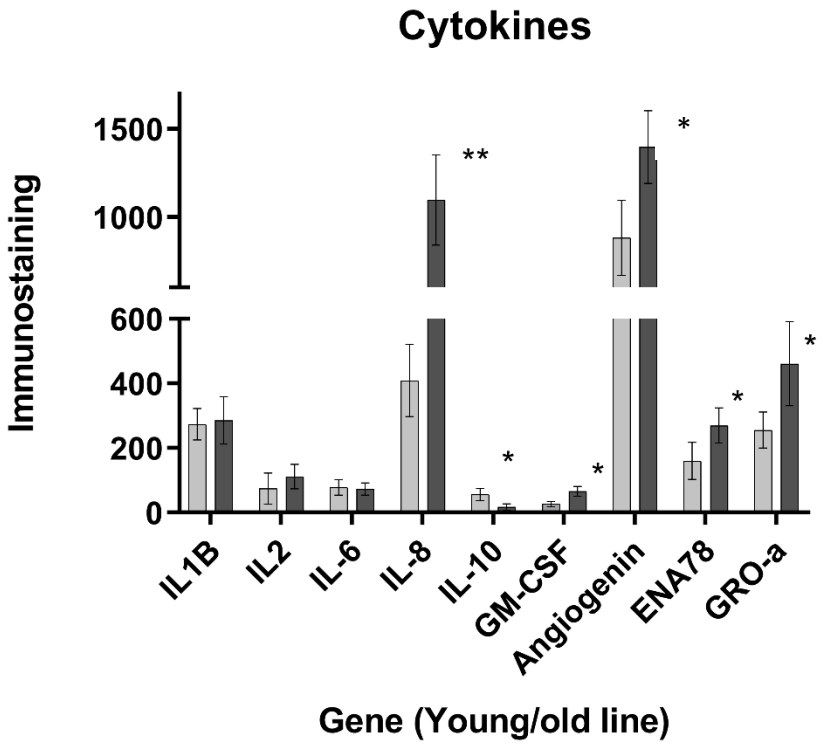

Figure 3.2 Key SASP cytokines as measured by ELISA

Figure displays data for key SASP cytokines based on those which demonstrate significant changes in the current research and those which have evidence of significance from previous research (79, 206). The changes observed in secreted cytokines in young (light grey bars) and old (dark grey bars) astrocytes (N=6). Statistically significant changes were notes in IL-8 ($P<0.005$) and IL-10, GM-CSF, Angiogenin, ENA78, GRO-a ($P<0.05$)

Table 3.2 Matrix Metalloproteinase levels in culture media of biologically old and young astrocytes

Table shows Gene ID, mean MMP secretion in young (Y) and old (O) astrocytes (N=6) in arbitrary units, standard deviation, log-base 2 of fold change. T values and P values. Statistically significant (P <0.05) changes are denoted by * and row is shown in bold typeface, those approaching but not meeting statistical significance are shown in green.

Gene	Mean Y	SD	Mean O	SD	Log2Fold Change	T	p
MMP-1	158.7	85.2	150.8	98.1	-0.07	-0.12	0.909
MMP-2	29.8	24.1	11.63	15.99	-1.36	-1.26	0.264
MMP-3	**75.9**	**14.98**	**128.3**	**30.8**	**0.76**	**3.06**	**0.038***
MMP-8	0.249	0.498	0	0		-	-
MMP-9	31.22	14.17	50.5	38.8	0.69	0.93	0.419
MMP-10	**6.28**	**6.29**	**18.57**	**5.15**	**1.56**	**3.02**	**0.029***
MMP-13	58.9	21.4	11.63	18.62	-2.34	2.37	0.064
TIMP-1	689.7	118	1074	291	0.64	2.45	0.091
TIMP-2	**4723**	**382**	**8403**	**1012**	**0.83**	**6.81**	**0.006***
TIMP-4	156.7	38.8	203.6	163.7	0.38	1.79	0.133

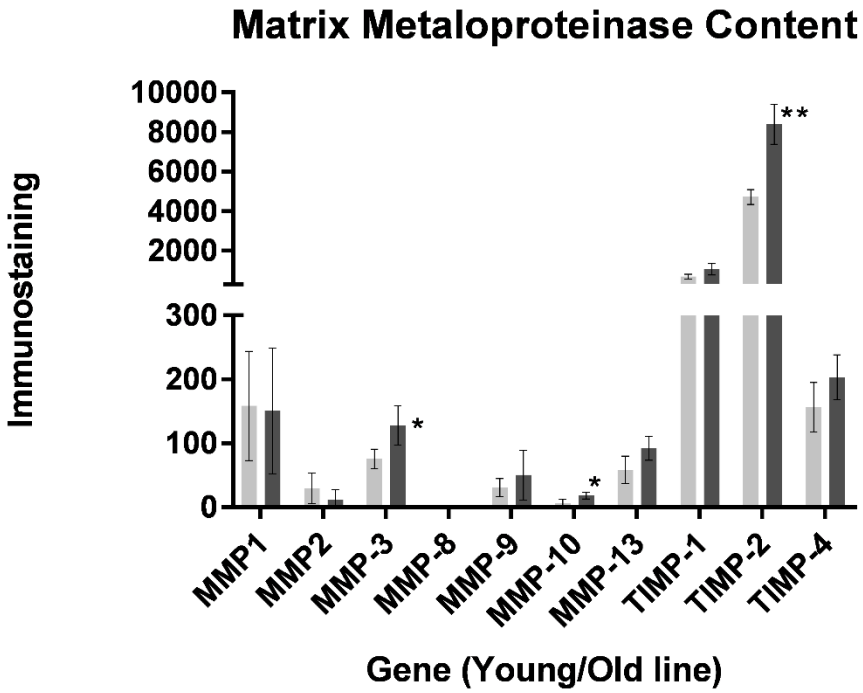

Figure 3.3 Matrix Metalloproteinase Content

MMP content in young vs old primary astrocytes (N=6). The light grey bar of each MMP represents the secreted MMP levels from young cells, and the dark grey bar of each colour represents the secreted MMP content from old cells. Statistically significant occurrences are marked with *.
* = $p < 0.05$, ** = $p < 0.005$.

3.1.2 Changes in splicing factor expression and patterns of alternative splicing in senescent astrocytes

We have previously demonstrated changes in splicing factor expression in senescent primary human cells of different lineages (33, 34, 37, 39). Similar changes were also apparent in senescent astrocytes, where 10/20 of the splicing factors tested demonstrated lower expression in late passage cells compared with earlier passage cells (Table 3.3, Figure 3.4). Both *HNRNP* splicing inhibitors and Serine-arginine (SR) rich splicing activator transcripts demonstrated dysregulation; 4/8 (50%) transcripts encoding splicing inhibitors, 3/8 splicing activator transcripts and 3/4 (75%) of core spliceosomal transcripts demonstrated changed expression in senescent astrocytes. Most genes selected for study were expressed in astrocytes; only *AQP4M1*, *EAAT2A* and *EAAT2B* were not. 8 of the remaining 13 transcripts demonstrated changes to their splicing patterns (Table 3.4, **Error! Reference source not found.**).

Table 3.3 Comparison of splicing factor levels in senescent and non-senescent astrocytes

Values given refer to the mean expression of each splicing factor in either early or late passage cells (N=6). Values in parentheses are the standard error of the mean. Splicing factors demonstrating significant differences in expression are given in bold italic typeface. 3 biological and 3 technical replicates per sample.

Splicing Factor	Early passage astrocytes	Late passage astrocytes	p-value
AKAP17A	1.035 (0.090)	1.01 (0.044)	0.077
HNRNPA0	***1.024 (0.077)***	***0.65 (0.031)***	***0.005***
HNRNPA1	1.236 (0.340)	1.863 (0.416)	0.449
HNRNPA2B1	1.343 (0.353)	0.6 (0.108)	0.257
HNRNPD	***1.094 (0.180)***	***0.646 (0.048)***	***0.03***
HNRNPH3	***1.071 (0.130)***	***0.743 (0.037)***	***0.003***
HNRNPK	1.153 (0.176)	1.37 (0.209)	0.551
HNRNPM	***1.021 (0.068)***	***0.66 (0.067)***	***0.012***
HNRNPUL2	1.074 (0.148)	0.784 (0.106)	0.089
IMP3	***1.088 (0.160)***	***0.62 (0.11)***	***0.035***
LSM14A	***1.042 (0.113)***	***0.315 (0.047)***	***0.005***
LSM2	1.377 (0.426)	0.369 (0.045)	0.065
PNISR	***1.041 (0.098)***	***0.777 (0.030)***	***0.005***
SF3B1	***1.018 (0.070)***	***0.716 (0.030)***	***0.018***
SRSF1	1.06 (0.140)	0.885 (0.038)	0.065
SRSF2	1.038 (0.095)	1.017 (0.045)	0.121
SRSF3	1.077 (0.175)	0.697 (0.032)	0.095
SRSF6	1.07 (0.126)	0.762 (0.104)	0.098
TRA2B	***1.022 (0.081)***	***0.757 (0.057)***	***0.031****
SRSF7	***1.119 (0.198)***	***0.51 (0.063)***	***0.015****

Table 3.4 Comparison of alternative isoforms of selected brain or senescence genes in senescent and non-senescent astrocytes

Values given refer to the mean expression of each splicing factor in either early or late passage cells (N=6). Values in parentheses are the standard error of the mean. Transcripts demonstrating significant differences in expression as measured by *t* test are given in bold italic typeface, with p-values in right hand column. 3 biological and 3 technical replicates per sample.

Transcript	Early passage astrocytes	Late passage astrocytes	p-value
GFAP(A)	***0.809(0.208)***	***0.062(0.013)***	***0.0021****
SKLOTHO	1.155(0.209)	0.498(0.275)	0.131
MKLOTHO	***1.059(0.122)***	***0.381(0.012)***	***0.005****
AQPM23	1.252(0.459)	1.067(0.434)	0.784
TAU3	***1.044(0.114)***	***0.603(0.043)***	***0.023****
TAU4	1.23(0.674)	1.542(0.196)	0.679
PSEN2	***1.009(0.12)***	***1.921(0.158)***	***0.01****
CDKN2A-p14^{ARF}	***1.043(0.044)***	***0.532(0.048)***	***0.001*****
CDKN2A-p16^{Ink4A}	***1.061(0.076)***	***2.133(0.127)***	***0.002****
p21a	***1.079(0.085)***	***1.666(0.087)***	***0.009****
p21b	***1.016(0.095)***	***2.041(0.077)***	***0.001*****
TP53	0.974(0.052)	1.038(0.082)	0.541
ATM	0.926(0.097)	0.612(0.069)	0.057

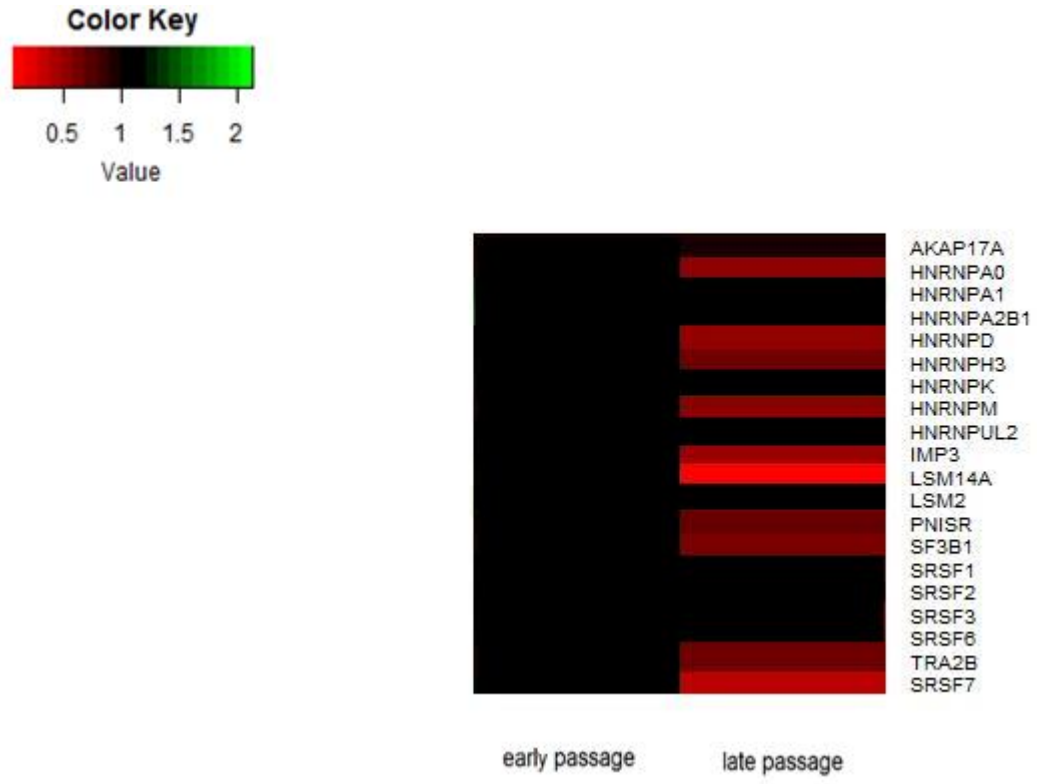

Figure 3.4 Heat map showing changes in splicing factor transcription between early and late passage astrocytes (N=6)

Non-statistically significant findings are blacked out. *HNRNPA0, HNRNPD, HNRNPH3, HNRNPM, IMP3, LSM14A, PSISR, SF3B1, TRA2B, SRSF7*, all displayed statistically significant changes in expression. Notably, most were downregulated, in line with current understanding suggesting that splicing factor expression decreases with age.

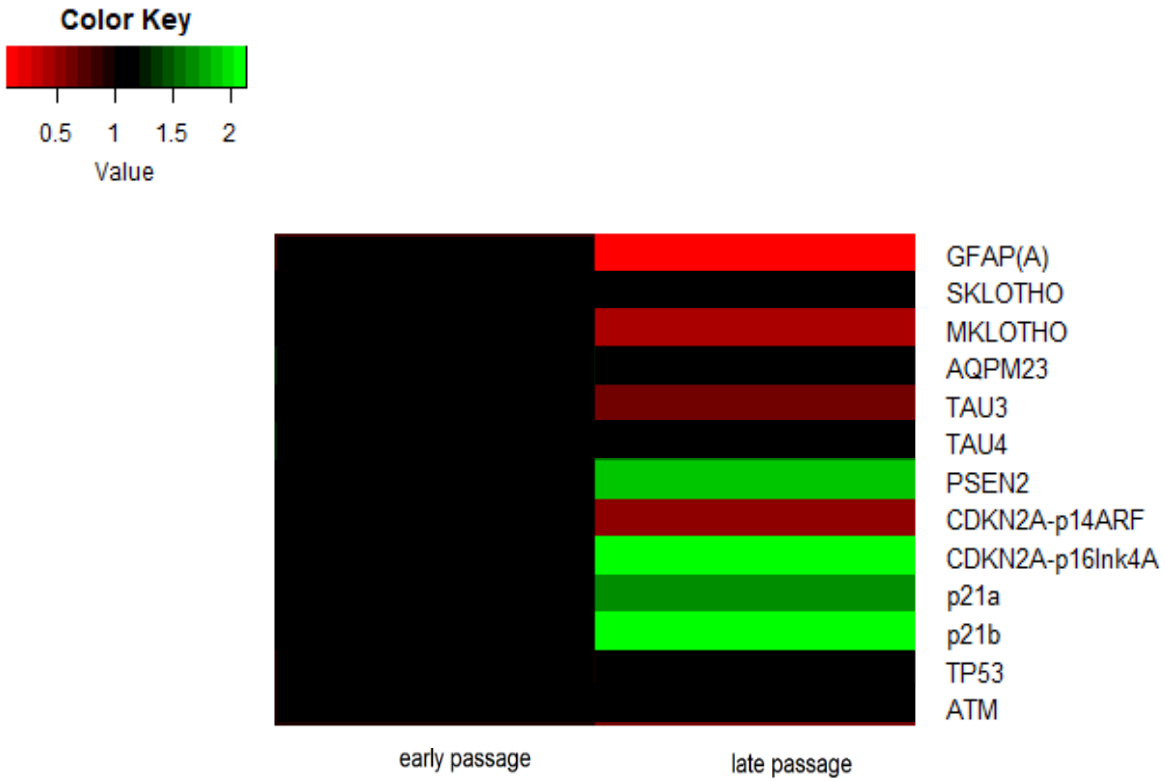

Figure 3.5 Isoform expression in young vs old astrocytes (N=6).

Heat map showing changes in isoform transcription between early and late passage astrocytes (N=6). Non-statistically significant findings are blacked out. Significant changes in isoform balance were observed in 8/13 mRNA species. Half of the transcripts experiencing significant changes were down regulated: *GFAP(A), mKlotho, TAU3* (all related to astrocyte function) and *P14* (cell cycle). We saw significant upregulation of 3 tumour-suppressive genes: *P16*, *P21a* and *P21b*, alongside *PSEN2. GFAPa mKlotho, Tau3* and *P14* had decreased expression.

3.1.3 Association of senescence-related transcripts with cognitive decline in a longitudinal human population

We next assessed whether any of the transcripts demonstrating senescence-related changes in aged primary human astrocytes were associated with cognitive decline as assessed by change in MMSE score between FU3 and FU4, in peripheral blood mRNA from individuals in the InCHIANTI study of aging. Of the 8 transcripts demonstrating associations with senescence in late passage astrocytes,

TAU3, *GFAPα*, m*KLOTHO CDKN2A(p14^{ARF}), CDKN2A(p16^{INK4A}), CDKN1A(p21a), CDKN1A(p21b) and PSEN2* were also expressed in peripheral blood, and suitable for ongoing analysis. *CDKN2A (p14^{ARF}), and TAU3* were positively associated with mild cognitive decline (*CDKN2A (p14^{ARF})* beta coefficient 0.122, 95% CI 0.01 to 0.24; p = 0.04, *TAU3* beta coefficient 0.170, 95% CI 0.042 to 0.297; p = 0.01) whereas *GFAPα* was negatively associated with mild cognitive decline (beta coefficient -0.196 (95% CI -0.36 to 0.032; p = 0.02; figure 6; table 8). Interestingly the only association we found with severe cognitive decline was a negative association with *TAU3* (beta coefficient -0.286, 95% CI -0.56 to 0.04; p = 0.04. *GFAPα* and *TAU3* demonstrated significant differences in level of isoform expression between mild and severe cognitive decline (Figure 3.6) (Table 3.5).

Table 3.5 Association between blood-based isoform production, induced by alternative splicing and cognitive decline in participants from the InCHIANTI population study of Aging

The table gives the beta coefficients, 95% confidence intervals (95% CI) and p-values for the association between candidate transcript expression and cognitive decline as assessed by change in MMSE for 197 individuals in a 3-7 year period. Mild cognitive decline is categorised as a decline of between 2 and 8 points in MMSE between FU3 and FU4, whereas severe cognitive decline is characterised as a decline of between 9 and 22 points. Transcripts demonstrating significant differences in expression are given in bold italic typeface.

Mild cognitive decline (2 – 8 point decline in MMSE)				**Severe cognitive decline (9 – 22 point decline in MMSE)**				**Difference between mild and severe decline in MMSE**			
Isoform	**Beta**	**95% CI**	**p-value**	**Isoform**	**Beta**	**95% CI**	**p-value**	**Isoform**	**Beta**	**95% CI**	**p-value**
GFAPα	***-0.196***	***-0.36 to 0.03***	***0.02***	*GFAPα*	0.129	-0.23 to 0.49	0.48	***GFAPα***	***0.363***	***0.08 to 0.65***	***0.01***
mKLOTHO	-0.032	-0.16 to 0.09	0.61	*mKLOTHO*	0.070	-0.20 to 0.34	0.61	*mKLOTHO*	0.093	-0.18 to 0.37	0.50
p14	***0.122***	***0.01 to 0.24***	***0.04***	*p14*	0.209	-0.04 to 0.46	0.10	*p14*	0.069	-0.18 to 0.32	0.59
P16	-0.097	-0.29 to 0.09	0.31	*P16*	-0.095	-0.51 to 0.32	0.65	*P16*	-0.029	-0.45 to 0.40	0.89
P21b	-0.015	-0.11 to 0.08	0.74	*P21b*	-0.085	-0.29 to 0.12	0.40	*P21b*	-0.030	-0.22 to 0.16	0.75
PSEN2	-0.075	-0.23 to 0.08	0.33	*PSEN2*	-0.215	-0.55 to 0.12	0.20	*PSEN2*	-0.133	-0.47 to 0.20	0.43
TAU3	***0.170***	***0.04 to 0.30***	***0.01***	***TAU3***	***-0.286***	***-0.56 to -0.01***	***0.04***	***TAU3***	***-0.460***	***-0.75 to -0.17***	***<0.01***

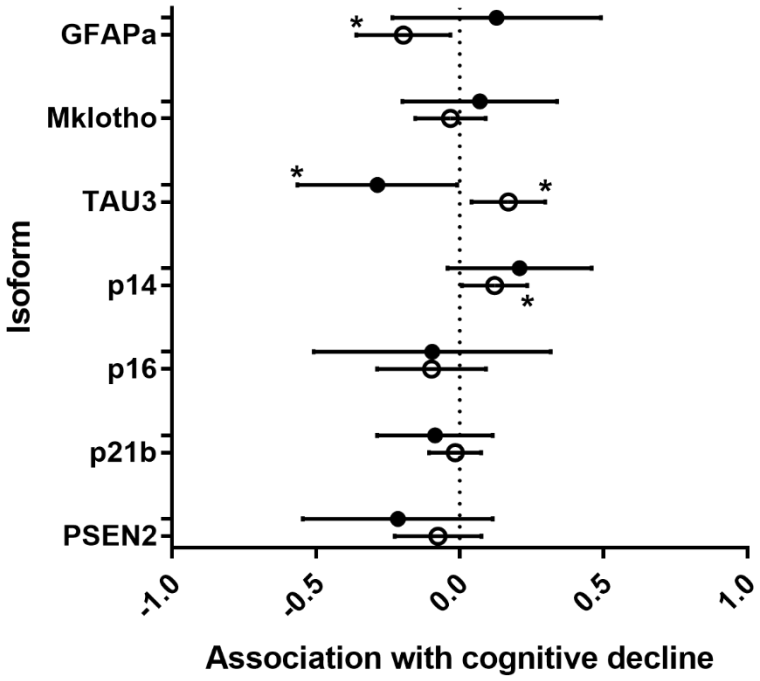

Figure 3.6 Forrest Plot of association with cognitive decline of specific Isoforms in the InCHIANTI study of Aging

The graph indicates the associations between peripheral blood expression of alternatively expressed transcripts of genes with known links with neurodegenerative disease or cellular senescence and mild or severe cognitive decline is given in this figure. Mild decline is denoted by open circles, severe decline is denoted by closed circles. * = $p < 0.05$. The null association point is given by the dotted line. Beta coefficients of association are given on the X axis and transcript identity are given on the Y axis. Data are from 197 participants classified as either mild cognitive decline (a reduction of 2-8 points in MMSE performance over a 3-7 year period) or severe decline (a reduction of 9-22 points in MMSE performance over the same period).

4 DISCUSSION

The accumulation of senescent cells due to repeated cell stresses is thought to be a major contributor to ageing and age-related disease. The ablation of senescent cells can bring about improvements in multiple age-related phenotypes in animal models (15, 17). Furthermore, correlations exist between circulating levels of senescence markers such as $p16^{INK4a}$ and HMGB2 and functional status in humans (207). Suppression of senescent cell characteristics such as SASP using the flavonoid apigenin has also been demonstrated to reduce aggressive behaviour in breast cancer cells, providing further evidence that reduction of senescent cell load may bring benefits for age-related diseases (208). To date, there have been a limited recorded trials of senolytics in humans (209, 210), although each with positive results. In September 2019, it was finally demonstrated that senolytics could clear senescent cells from the bodies of humans (211).

Whilst the evolution of cellular senescence is understood to have emerged in part because of the tumour suppressive properties of the cell state, the senescence associated secretory phenotype has deleterious properties and the inflammatory paracrine effect can drive tumorigenesis in surrounding tissues. Therefore, whilst the individual cell may use the senescence pathway to escape neoplasm formation, the relief is only a temporary one. Senescent cell development triggers a chain reaction, propagating dysfunction through tissues by inflammation. This tumour suppressive "side-effect" appears to be a typical example of evolution precipitating antagonistic pleiotropic effects, commonly associated with ageing (212).

Based on evidence linking senescence to RNA processing, we hypothesised that senescent astrocytes would display differential expression of splicing regulatory factors and altered patterns of alternative splicing *in vitro*. We also suggested that due to the systemic nature of ageing, or the blood brain barrier

degradation over time, some of these isoform changes may be detectable in peripheral blood and show statistical associations with cognitive phenotypes in human populations.

Initially were able to show that replication competent, primary human astrocytes in culture do indeed reach a senescent state, simply through continued passage, and we generated evidence for a direct link between this and the reduction in splicing faction expression, demonstrably critical to normal transcriptional regulation and response to environmental stress. We also have found functional evidence that the expression changes of splicing factors in a highly senescent cell population appear to be able to drive imbalances in isoform species known to be highly important in astrocyte function. What is not yet clear, is if senescence drives dysregulation, if dysregulation drives senescence, or if in fact they are both 'symptoms' of some other event.

As such, age-related dysregulation of alternative splicing may contribute to or be a result of the accumulation of senescent cells during ageing. It would be of importance to ascertain if re-regulation of splicing in this cell line using small molecule effectors of splicing is possible, and if this can then modify the senescence state. Restoration of splicing factor expression can reverse cellular senescence and bring about rejuvenation of multiple other cell types in culture.

A look at the *in-vitro* results of isoform expression demonstrates results congruent with the senescence hypothesis, and with evidence available in existing literature regarding the expression of tumour suppressors P53, P16, and P21a. Specifically, we observed a robust increase in the expression of P16, P21a and P53 isoforms overall in an aged cell population as seen elsewhere (213). Even more encouraging was the observation that P14 expression, which produces a protein known to be associated with cell cycle arrest and premature senescence was positively associated with cognitive decline in our population study, providing potential biomarker for the pathology or even perhaps therapeutic targets.

We demonstrated that 50% of splicing factor transcripts we measured demonstrated dysregulated expression in senescent astrocytes, and splicing changes were evident for almost half of the alternatively spliced genes in an *a priori* panel of candidate genes. The changes in astrocyte splicing factor and isoform expression we have identified here, likely do not act directly on the transcriptome observable in whole blood, but rather reflect related cognition-associated changes to systemic splicing factor expression; changes which are also observable in blood, as we have recently described (35)

8/13 candidate isoforms demonstrated splicing alterations in senescent cells and were also expressed in human peripheral blood. Of these, 3 (*TAU3, GFAPα,* and *CDKN2A (p14^{ARF}*) were associated with mild cognitive decline in ageing humans. The *TAU3* isoform was also associated with severe cognitive decline, but in an opposing direction. Our data are consistent with a model whereby age-related splicing factor changes may lead to splicing patterns for genes with roles in brain function or senescence, which may influence the development of cognitive decline in the human population. The links between splicing factor changes and isoform changes are impossible to predict from global measures. Each individual splice site in each gene is regulated by a unique and specific combination of activators and inhibitors which determine its usage or not, which makes prediction impossible from levels alone. What our data do indicate is that senescent astrocytes have disrupted expression of many splicing factors, which would be predicted to alter the splicing patterns. We have demonstrated that this holds true for several genes important in senescence or in astrocyte function.

The presence of senescent cells has been suggested to contribute to shortened overall lifespan (16), and clearance of such cells was able to bring about a delay in the appearance of ageing phenotypes in ageing mice (17). Other groups have reported increased lifespan, rejuvenation of ageing phenotypes such as thinning fur and improved kidney function in old mice that have undergone targeted removal of senescent cells (214). Ablation of senescent glial cells has been demonstrated to lead to a reduction of Tau-dependent pathology and improve cognitive function in mice (23). Accordingly, we observe the

generation of a strong SASP in astrocytes that have undergone replicative senescence, which may be contributory to the inflammatory increases evident in the pathophysiology of neurodegeneration. The SASP profile exhibited by the primary human astrocytes does not match that profile seen in other cell lines from our previous work (34, 37, 39), however cell type specificity in SASP has previously been described (215). The reasons behind these variations are unclear, but may reflect histologically discrete programmes, dysregulation patterns specific to existing transcriptomic profiles, or simply arbitrary patterns of cytokine production which represent the stochastic molecular dysregulation which is occurring.

Unpicking the specific components of the SASP is no easy task. Investigation into existing literature does demonstrate astrocyte specific IL-8 secretion during times of stress such as acidosis (216). IL-8 has neuroprotective and neurotrophic effects and is an angiogenesis promotor. It drives production of brain derived neurotrophic factor and seems to prevent amyloid beta induced apoptosis in Alzheimer's. IL-8 was found to be much higher concentrations in the CSF of individuals with mild cognitive impairment as opposed to those with Alzheimer's (217).

Our observations, together with the marked changes in splicing factor expression are suggestive that the intersection between disrupted regulation of splicing, cellular senescence and its associated inflammatory phenotype in astrocytes may contribute to astrocyte dysfunction and have some bearing on eventual cognitive decline. Similar changes may also be occurring in other important proliferative brain cell types such as microglia.

Similarly, the altered expression of alternatively expressed transcripts in blood may be reflective of changes that we demonstrate here also occur in astrocytes. We identified positive associations between *CDKN2A (p14^{ARF}), and TAU3* expression in peripheral blood and mild cognitive decline, and a negative correlation between *GFAPα* expression and mild cognitive decline. The associations between

GFAPA and *CDKN2A (p14^{ARF})* were not apparent in severe cognitive decline, although this may reflect low power imposed by the inherent variability of human biological samples, since p14 expression in severe decline is trending in the same direction.

The association of *TAU3* expression with severe cognitive decline was still evident, but was negatively, rather than positively correlated. GFAPα expression also demonstrates opposing direction of effect between mild and severe decline. This may represent differences in cell subtype populations or altered cell characteristics between disease states. GFAPα, which along with other intermediate filaments forms the cytoskeleton is important for signal transduction and structural properties (218). *GFAPα* is highly expressed during key developmental stages during gestation, and has also been observed to have elevated expression levels in brain damage and a range of neurological diseases (218). Some studies have reported no change in whole blood GFAPα protein levels, and hypothesised that without rapid astroglial destruction, GFAPα levels may not climb to a detectable level and thus may not be a good indicator of neurological pathology (219). Such studies however may not detect changes at the level of isoforms, if appropriate antibodies capable of specifically identifying individual splice variants are not used.

Increased levels of *CDKN2A (p14^{ARF})* may be reflective of an increased load of senescent cells, and is one of two proteins produced by alternate reading frame of the *CDKN2A* locus (220). p14^{ARF} inhibits the activity of MDM2, a protein which sequesters the p53 protein (221). Once p53 stabilises and accumulates it can trigger DNA repair or the apoptosis program of cell death (222). The consequences of increased p14^{ARF} levels are complex and in places conflicted. It has been demonstrated to induce either cellular senescence or apoptosis in a *p53* dependant manner (220). There have also been some reports that although *CDKN2A (p14^{ARF})* transcripts are upregulated in senescent cells, *TP53* and *MDM2* levels can remain unchanged (221). Ectopic expression of p14^{ARF} is capable of inducing senescence but overexpression must be maintained to commit cells to a senescent state (223).

TAU3 transcript expression in the blood was observed to be correlated positively with mild cognitive decline, but interestingly, negatively correlated with severe cognitive decline. Microtubule associated protein Tau (*MAPT*) is an alternatively spliced regulator of microtubule dynamics – which is essential for cellular functions from structure to transport (224). An ever growing body of research in human and animal models of tauopathies is demonstrating that the delicately balanced 2:1 3R/4R TAU ratio (225), which is achieved through alternative splicing of exon 10, is imperative for maintaining healthy function in cells in the brain, and shifts to disrupt this balance in either direction can be catastrophic (225-228). A number of neurodegenerative diseases, including Alzheimer's and frontotemporal dementia (FTD), have had tau isoform balance (specifically 3R and 4R isoforms) implicated in their pathophysiology (224, 229, 230). These include those examples with premature cognitive decline (231). The precise mechanisms by which TAU isoforms specifically contribute to each neurological pathology are still being investigated. The directionality of association between *TAU3* transcript levels and mild and severe decline are consistent with existing literature suggesting imbalances in 3R/4R isoform ratios, rather than absolute isoform levels per se, are identified in cases of dementia and examples of neurodegenerative disease (225, 232).

Our finding of dysregulated splicing factor expression in human primary astrocytes is novel, and consistent with reports from senescent cells of other tissue types (33, 34, 37, 39). Our study benefits from a systematic cells-to-populations approach including primary human cell lines and an exquisitely characterised longitudinal population study. Weaknesses of this study are the initial assessment in isolated astrocytes, which may not represent the holistic nature of the cytological and molecular mechanisms involved in cognitive decline and may not capture the extent of cross talk between other cell types in the human brain. It also needs to be recognised that the astrocytes used in this study derive from a single donor, and that differences reported here would benefit from confirmation in individuals of different genetic background. Cell lines from a wider variety of donors may return more genes of interest, however these tissues are rare, and difficult to acquire. In addition, our *a priori* list

of genes do not include many other isoforms and genes involved in the process. Several of the isoforms selected for study are also not strictly specific for astrocytes. This is particularly true of the senescence genes tested, which reflect players in a more global mechanism. Our scope was narrowed by the need for these genes to be expressed in the blood, for assessment of association with living population. Further work may benefit from whole transcriptome sequencing and transcriptional profiling in several different cell types.

Our data are consistent with a model by which accumulation of senescent astrocytes (and doubtless other important brain cell subtypes), their associated disrupted splicing patterns and the increased inflammatory microenvironment may contribute to premature cognitive decline. Inflammation and related cellular stresses are capable of activating cell signalling pathways and lead to further dysregulation of splicing factor expression (38), so it is possible that an auto-regulated feedback loop involving SASP-derived increases in inflammation, dysregulated splicing regulation and subsequent further increases in senescent cell load may occur as a result of positive feedback. The idea that some of the features of cognitive decline could therefore arise from dysregulated splicing of genes important in the support cells of the brain requires further exploration. This could be explored further by the selective manipulation of specific isoform levels, followed by assessment of effects on astrocyte cell kinetics or astrocyte function in cells and in systems. Our observations suggest that the role of splicing factor expression and dysregulated alternative splicing in cognitive decline may represent an interesting line of future investigation.

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