



LUND UNIVERSITY

The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease

Schultz, Nina

2018

Document Version:

Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):

Schultz, N. (2018). *The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Malmö]. Lund University: Faculty of Medicine.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

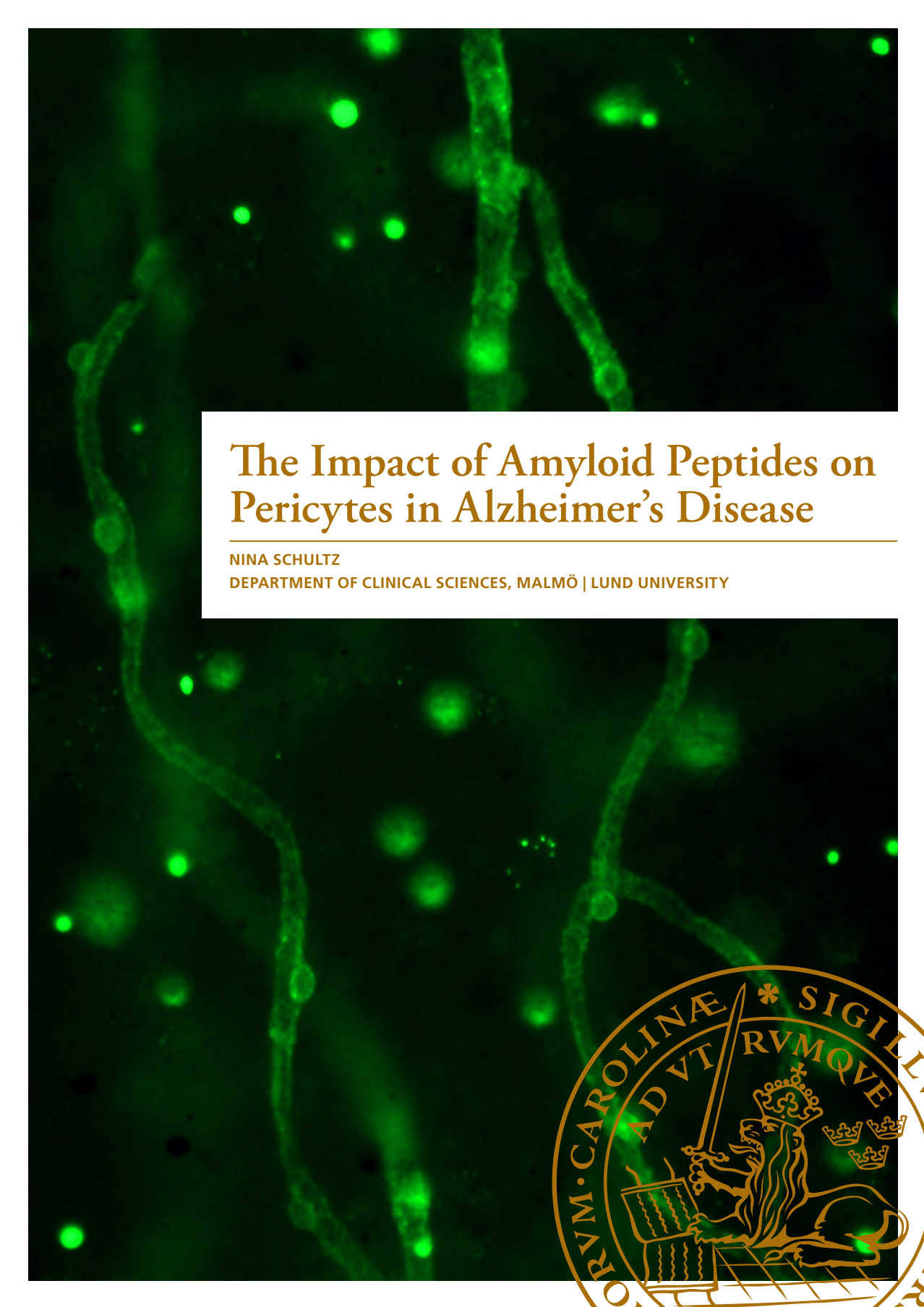
Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

A fluorescence microscopy image showing a network of green, thread-like structures (likely amyloid peptides) and several bright green, circular spots (likely pericytes) against a dark background.

The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease

NINA SCHULTZ

DEPARTMENT OF CLINICAL SCIENCES, MALMÖ | LUND UNIVERSITY



The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease

Nina Schultz



LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended December 14th 2018 at 09.00 in Lilla Aulan, Medicinskt forskningscentrum, Jan Waldenströmsgata 5, Malmö.

Faculty opponent

Professor Roxana Carare, University of Southampton

Organization LUND UNIVERSITY Clinical Memory Research Unit Department of Clinical Sciences, Malmö Faculty of Medicine Author(s) Nina Schultz	<table border="1"> <tr> <td data-bbox="564 125 799 179"> Document name Doctoral dissertation </td> <td data-bbox="806 125 1132 179"></td> </tr> <tr> <td data-bbox="564 179 799 250"> Date of issue 14th of December 2018 </td> <td data-bbox="806 179 1132 250"></td> </tr> <tr> <td data-bbox="564 250 799 301"> Sponsoring organization SWEAH </td> <td data-bbox="806 250 1132 301"></td> </tr> </table>		Document name Doctoral dissertation		Date of issue 14 th of December 2018		Sponsoring organization SWEAH	
Document name Doctoral dissertation								
Date of issue 14 th of December 2018								
Sponsoring organization SWEAH								
Title and subtitle The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease								
Abstract <p>Background: The neurodegenerative disorder Alzheimer's disease (AD) is neuropathologically characterized by hyperphosphorylated tau protein and extracellular depositions of amyloid beta (Aβ) peptides. In addition, patients with AD often also display vascular alterations such as impaired blood-brain barrier function, aberrant angiogenesis and loss of pericytes, a vessel-supporting cell type crucial for vessel integrity. The exact mechanism underlying the pericyte loss is not fully known but degenerative pericytes have been found adjacent to vessel-associated depositions of Aβ, suggesting this peptide to be toxic for pericytes. Interestingly, another amyloid peptide has recently been shown to form depositions in brain vessels of AD patients. This peptide, called islet amyloid polypeptide (IAPP), is foremost associated with type 2 diabetes (T2D) since it is believed to underlie the T2D characteristic β-cell death. Whether IAPP is toxic also for pericytes has not been investigated.</p> <p>Aim: To investigate how amyloid peptides such as Aβ and IAPP influence pericyte function in Alzheimer's disease.</p> <p>Methods: Pericyte alterations in the presence of amyloid peptides (Aβ1-40, Aβ1-42 and IAPP) were analyzed in human post-mortem tissue (brain and retina) (Papers I, III and IV) and human primary pericyte cultures (Papers I, II and III) by the use of immunostainings and immunoassays.</p> <p>Results: I) Patients with AD displayed reduced number of hippocampal pericytes and the number of pericytes was foremost associated with levels of Aβ1-40, and not Aβ1-42. In vitro studies further demonstrated both species- and aggregation-dependent differences in toxicity and proliferation. II) Aβ1-42 affected pericytic NG2 shedding in an aggregation-dependent manner with increased NG2 shedding after oligomer Aβ1-42 treatment and reduced NG2 shedding after treatment with fibrillar Aβ1-42. Furthermore, oligomer Aβ1-42-induced NG2 shedding was mediated via MMP-9. III) Human brain tissue studies showed intracellular inclusions of IAPP within pericytes that also displayed apoptotic features. In vitro studies demonstrated internalization of IAPP, which induced an aggregation-dependent toxicity, with the strongest response after treatment with oligomer IAPP. IV) Changed levels of IAPP and a reduced number of pericytes were found in retina of AD patients. Total levels of unmodified hippocampal IAPP, high-molecular unmodified IAPP and pericyte numbers were in controls shown to correlate with same variables in retina. In AD patients, instead a correlation was found between levels of hippocampal high-molecular total (unmodified and modified) IAPP and retinal high-molecular total IAPP.</p> <p>Conclusions: This thesis supports previous studies demonstrating pericyte loss in AD patients. Moreover, amyloid peptides associated with AD (Aβ1-40, Aβ1-42 and IAPP) influence pericyte proliferation and survival in specie- and aggregation dependent manner. These studies highlight amyloid peptides as potential regulators of the pericyte population in the healthy and diseased brain.</p>								
Key words Pericytes, Alzheimer's disease, amyloid peptides, A β 1-40, A β 1-42, IAPP, cell death, NG2, retina, hippocampus								
Classification system and/or index terms (if any)								
Supplementary bibliographical information	Language English							
ISSN and key title 1652-8220	ISBN 978-91-7619-708-0							
Recipient's notes	Number of pages 106	Price						
	Security classification							

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature



Date 2018-11-08

The Impact of Amyloid Peptides on Pericytes in Alzheimer's Disease

Nina Schultz



LUND
UNIVERSITY

Cover photo taken by Nina Schultz: *NG2 staining of retinal pericytes and vessels.*

Copyright pp 1-106 Nina Schultz

Paper 1 © Open Access

Paper 2 © Open Access

Paper 3 © Open Access

Paper 4 © Open Access

Clinical Memory Research Unit
Department of Clinical Sciences, Malmö
Faculty of Medicine
Lund University

ISBN 978-7619-708-0

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2018



MADE IN SWEDEN 

Media-Tryck is an environmentally
certified and ISO 14001 certified
provider of printed material.
Read more about our environmental
work at www.mediatryck.lu.se

To my grandmother Odette

Table of Contents

Abstract	9
List of publications.....	11
Abbreviations	12
Sammanfattning på svenska	15
1. Background	19
1.1 Alzheimer's disease	19
1.1.1 Clinical symptoms	19
1.1.2 Neuropathological manifestations	20
1.1.3 Vasculopathy in AD	22
1.1.4 Current diagnostics	22
1.1.5 Need for new diagnostic tools	23
1.1.6 Familial types of AD	23
1.1.7 Sporadic type of AD	24
1.2 Pericytes	25
1.2.1 Markers for pericyte identification	25
1.2.2 Component in blood brain barrier	26
1.2.3 Vessel stability and angiogenesis	27
1.2.4 Secretory properties	27
1.2.5 Neural/glial antigen 2	28
1.2.6 Pericytes in AD	29
1.3 Amyloid beta	30
1.3.1 APP cleavage and the formation of A β	30
1.3.2 A β 1-42	31
1.3.3 A β 1-40	32
1.3.4 Amyloid peptides and pericytes	32
1.4 Islet amyloid polypeptide (IAPP)	32
1.4.1 Kinetics	33
1.4.2 Physiological roles of biologically active IAPP	33
1.4.3 Aggregation and pathological roles of IAPP	33
1.4.4 The role of IAPP in AD	34
1.4.5 IAPP and pericytes	35
2. Aims of the thesis	37

3. Material and methods	39
3.1 Human post-mortem tissue samples.....	39
3.1.1 Human post-mortem brain and eye tissue	39
3.1.2 Homogenization of human brain and eye tissue.....	39
3.1.3 Immunohistochemical (IHC) procedures	40
3.1.4 Immunofluorescence (IF) procedures.....	40
3.1.5 Analysis of A β 1-40 and A β 1-42	42
3.1.6 Analysis of unmodified IAPP	42
3.1.7 Analysis of total (unmodified and modified) IAPP	42
3.1.8 Dot blot analysis of IAPP levels.....	42
3.1.9 Quantification of number of pericytes and vessel length	43
3.1.10 Quantification of NG2 immunoreactivity intensity	43
3.2 Cells.....	43
3.2.1 Cell cultures.....	43
3.2.2 Cell identification	44
3.2.3 Immunofluorescence (IF) procedures of cell cultures	44
3.2.4 Synthetic A β 42 preparations	44
3.2.5 Recombinant A β 40 and A β 42 preparations	45
3.2.6 Synthetic IAPP preparations.....	45
3.2.7 Oligomer analysis	45
3.2.8 Fibril analysis	46
3.2.9 Transmission electron microscopy (TEM) analysis	46
3.2.10 Western blot analysis.....	46
3.2.11 Cell treatment	47
3.2.12 Cell cytotoxicity tests	47
3.2.13 Proliferation assay using Ki67.....	48
3.2.14 NG2 analysis	48
3.2.15 MMP-9 and MMP-2 activity assay	48
3.2.16 MMP-1/3/9 analysis	48
3.2.17 Autophagy	49
3.2.18 IAPP internalization	49
3.2.19 Microscopy	49
3.3 Statistical analysis	49
4. Main results.....	51
4.1. Paper I	51
4.1.1. Brain tissue studies	51
4.1.2. Cell culture studies	53
4.1.3. Conclusion Paper I	55
4.2. Paper II	55
4.2.1. Cell culture studies	56
4.2.2. Conclusion Paper II	57

4.3 Paper III.....	58
4.3.1 Brain tissue studies	58
4.3.2. Cell culture studies	59
4.3.3. Conclusion Paper III.....	62
4.4. Paper IV	62
4.4.1. Hippocampal and retinal tissue.....	62
4.4.2 Conclusion Paper IV.....	66
5. Discussion	67
5.1 Results in context	67
5.1.1 The impact of A β 1-42 on pericytes	67
5.1.2 The impact of A β 1-40 on pericytes	69
5.1.3 The impact of IAPP on pericytes.....	71
5.2 Methodological considerations	73
5.2.1 Post-mortem study limitations- cohort heterogeneity	74
5.2.2 Post-mortem study limitations- immunohistochemistry.....	75
5.2.3 In vitro model limitations - applicability	76
5.2.4 In vitro model limitations – peptide preparations.....	77
5.3 Implications and future directives	78
5.3.1 Diagnostic tools	79
5.3.2 New drug targets.....	80
6. Conclusions	81
7. Acknowledgements	83
8. References	87

Abstract

Background: The neurodegenerative disorder Alzheimer's disease (AD) is neuropathologically characterized by hyperphosphorylated tau protein and extracellular depositions of amyloid beta (A β) peptides. In addition, patients with AD often also display vascular alterations such as impaired blood-brain barrier function, aberrant angiogenesis and loss of pericytes, a vessel-supporting cell type crucial for vessel integrity. The exact mechanism underlying the pericyte loss is not fully known but degenerative pericytes have been found adjacent to vessel-associated depositions of A β , suggesting this peptide to be toxic for pericytes. Interestingly, another amyloid peptide has recently been shown to form depositions in brain vessels of AD patients. This peptide, called islet amyloid polypeptide (IAPP), is foremost associated with type 2 diabetes (T2D) since it is believed to underlie the T2D characteristic β -cell death. Whether IAPP is toxic also for pericytes has not been investigated.

Aim: To investigate how amyloid peptides such as A β and IAPP influence pericyte function in Alzheimer's disease.

Methods: Pericyte alterations in the presence of amyloid peptides (A β 1-40, A β 1-42 and IAPP) were analyzed in human post-mortem tissue (brain and retina) (**Papers I, III and IV**) and human primary pericyte cultures (**Papers I, II and III**) by the use of immunostainings and immunoassays.

Results: I) Patients with AD displayed reduced number of hippocampal pericytes and the number of pericytes was foremost associated with levels of A β 1-40, and not A β 1-42. In vitro studies further demonstrated both species- and aggregation-dependent differences in toxicity and proliferation, where only the fibril form of A β 1-40, and not oligomer A β 1-40 or any aggregation forms of A β 1-42, induced pericyte death and reduced proliferation. On the other hand, monomers of A β 1-40 increased cell viability and proliferation. **II)** A β 1-42 affected pericytic NG2 shedding in an aggregation-dependent manner with increased NG2 shedding after oligomer A β 1-42 treatment and reduced NG2 shedding after treatment with fibrillar A β 1-42. Furthermore, oligomer A β 1-42-induced NG2 shedding was mediated via MMP-9. **III)** Human brain tissue studies showed intracellular inclusions of IAPP within pericytes that also displayed apoptotic features. In vitro studies demonstrated internalization of IAPP, which induced an aggregation-dependent toxicity, with the strongest response after treatment with oligomer IAPP. **IV)** Changed levels of IAPP and a reduced number of pericytes were found in retina of AD patients. Total levels of unmodified hippocampal IAPP, high-molecular unmodified IAPP and pericyte numbers were in controls shown to correlate with same variables in retina. In AD patients, instead a correlation was found between levels of hippocampal high-

molecular total (unmodified and modified) IAPP and retinal high-molecular total IAPP.

Conclusions: This thesis supports previous studies demonstrating pericyte loss in AD patients. Moreover, amyloid peptides associated with AD (A β 1-40, A β 1-42 and IAPP) influence pericyte proliferation and survival in specie- and aggregation dependent manner. These studies highlight amyloid peptides as potential regulators of the pericyte population in the healthy and diseased brain.

List of publications

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals. All articles are found in the appendices. Reprints were made with the permission of the copyright owners.

- I.** Schultz N, Brännström K, Byman E, Moussaud S, Nielsen HM, The Netherlands Brain Bank, Olofsson A, Wennström M
Amyloid-beta 1-40 is associated with alterations in NG2+ pericyte population ex vivo and in vitro. *Aging Cell*. 2018 Jun;17(3)
- II.** Schultz N, Nielsen HM, Minthon L, Wennström M
Involvement of matrix metalloproteinase-9 in amyloid- β 1-42-induced shedding of the pericyte proteoglycan NG2. *J Neuropathol Exp Neurol*. 2014 Jul;73(7)
- III.** Schultz N, Byman E, Fex M, Wennström M
Amylin alters human brain pericyte viability and NG2 expression. *J Cereb Blood Flow Metab*. 2017 Apr;37(4):1470-1482.
- IV.** Schultz N, Byman E, The Netherlands Brain Bank, Wennström M
Levels of retinal IAPP are altered in Alzheimer's disease patients and correlate with vascular changes and hippocampal IAPP levels. *Neurobiol Aging*. 2018 Sep; 69:94-101.

Paper not included in this thesis

Byman E, Schultz N, The Netherlands Brain Bank, Fex M, Wennström M
Brain alpha-amylase: a novel energy regulator important in Alzheimer disease? *Brain Pathol*. 2018 Feb 27

Reprints were made with the permission of the copyright owners.

Abbreviations

A β	Amyloid beta
AD	Alzheimer's disease
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
α SMA	Alpha smooth muscle actin
BBB	Blood-brain barrier
BRB	Blood-retina barrier
BS	Blocking solution
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CD	Cluster of differentiation
CNS	Central nervous system
CO ₂	Carbon dioxide
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CVD	Cardiovascular disease
Ctrl	Control
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
DLB	Dementia with Lewy body
DMSO	Dimethyl sulfoxide
GuHCl	Guanidine hydrochloride
HBVP	Human brain vascular pericytes
HFIP	Hexafluoroisopropanol
HRP	Horse radish peroxidase
EC	Endothelial cell

ECL	Electrochemilumiscence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EP	Enriched preparations
FA	Formic acid
fAD	Familial AD
FAM	Fluorescein amidite
F	Fibril
FL	Fluorescence labelled
IAPP	Islet amyloid polypeptide/ amylin
ICAM-1	Intercellular Adhesion Molecule 1
IHC	Immunohistochemical
IPAD	Intramural periarterial drainage pathway
LB	Lewy body
LC3	Microtubule-associated protein-1 light chain 3
LC3-FP	Fluorescently labelled LC3
LDH	Lactate dehydrogenase
LRP-1	Lipoprotein receptor-related protein 1
M	Monomer
ML	Molecular layer
MMP	Matrix metalloproteinase
MMSE	Mini Mental State Examination
MOCA	Montreal Cognitive Assessment
MRI	Magnetic resonance imaging
NaOH	Sodium hydroxide
NBB	The Netherlands Brain Bank
NC	Non-demented control
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate

NG2	Neural/glial antigen 2
O	Oligomer
OMAB	Oligomer monoclonal antibody
ON	Overnight
OPC	Oligodendrocyte precursor cells
PBS	Phosphate-buffered saline
PDGF- β	Platelet derived growth factor beta
PDGFR- β	Platelet derived growth factor receptor beta
PHC	Parahippocampal cortex
PM	Pericyte medium
p-tau	Phosphorylated tau
P _{U-FA}	Pellet from ultra-centrifuged formic acid treated homogenate
P _{U-FA-G}	Pellet from ultra-centrifuged formic acid treated homogenate treated with GuHCl
PVDF	Polyvinylidene difluoride
RT	Room temperature
S _{L-FA}	Supernatant from low-speed centrifuged formic acid treated homogenate
sNG2	Soluble neural/glial antigen 2
S _{U-FA}	Ultra-centrifuged formic acid treated homogenate
T2D	Type 2 diabetes
TEM	Transmission electron microscopy
ThT	Thioflavin T
TMB	3,3',5,5'- Tetramethylbenzidine
t-tau	Total tau
vSMC	Vascular smooth muscle cells
WB	Western blot

Sammanfattning på svenska

Alzheimers sjukdom (AD) är den vanligaste förekommande formen av demens och år 2017 beräknades omkring 40 miljoner personer vara drabbade i världen. Personer som drabbas av AD uppvisar minnessvårigheter, försämrad rumsuppfattning och andra nedsättningar av tankeförmågor; symptom som beror på sjukliga förändringar i hjärnan. Vid obduktion syns en tydlig atrofi (vävnadsförlust) i stora delar av hjärnan, bland annat i områden viktiga för minnet. Om man tittar närmare på de drabbade områdena kan man se ytterligare förändringar, där de två vanligaste är trådliknande strukturer inuti nervceller (s.k. tangles) och extracellulära ansamlingar av proteinet amyloid beta ($A\beta$) (s.k. senila plack). Amyloid beta produceras i olika former i hjärnan, där $A\beta$ 1-42 och $A\beta$ 1-40 är de två vanligaste. Dessa peptiderna har i normala nivåer en viktig funktion i den friska hjärnan, men hos personer med AD ökar nivåerna markant, vilket leder till att peptiderna ansamlas och bildar aggregat, s.k. oligomerer och fibriller. Fibrillerna bildar i sin tur de AD-karaktäristiska senila placken.

Amyloid beta kan även ansamlas i och omkring hjärnans kärl; dels som perivaskulära plack (bestående huvudsakligen av $A\beta$ 1-42) men även som cerebral amyloid angiopathy (CAA), ett fenomen där $A\beta$ (framförallt $A\beta$ 1-40) ansamlas i kärlväggen. Kärl med sådana ansamlingar blir stela, instabila och börjar läcka. På vilket sätt $A\beta$ leder till dessa kärlförändringar vet man inte, men man har hittat döende pericyter, i nära anslutning till kärl med sådana ansamlingar. Pericyter är en kärlstabiliserande celltyp, viktig för nybildning av kärl och upprätthållandet av blod-hjärnbarriären. De spelar även en viktig roll i att föra bort skadliga substanser, såsom $A\beta$. Med tanke på deras viktiga funktioner skulle förlust av pericyter i hjärnan kunna få allvarliga konsekvenser med dysfunktionella kärl och en accelererad ansamling av skadligt $A\beta$ som följd. Denna hypotes har på senare år fått mycket uppmärksamhet inom AD-forskningsfältet, framförallt då man dessutom har funnit både vaskulära förändringar och färre pericyter hos personer med AD.

Nya studier har visat att ytterligare en amyloid peptid, utöver $A\beta$, kan vara viktigt i AD. Denna peptid kallas islet amyloid polypeptide (IAPP) och utsöndras tillsammans med insulin från bukspottskörteln. IAPP är normalt involverad i ämnesomsättningen och regleringen av aptit, men associeras ibland med typ 2 diabetes (T2D) eftersom ansamlingar av IAPP ofta ses i bukspottskörteln och andra organ hos dessa patienter. Ansamlingar av IAPP är toxiska och har satts i samband

med de insulinproducerande cellernas (β -celler) dysfunktion som karakteriserar sjukdomen. Intressant är även att IAPP även visat sig ansamlas i hjärnans blodkärl hos personer med demens och samtidig T2D samt hos personer med AD. Huruvida celler i kärlväggen påverkas av IAPP på samma sätt som bukspottskörtelns β -celler är ännu inte studerat, men på grund av likheterna mellan IAPP och A β , är det inte omöjligt att IAPP är skadlig även för pericyter.

Målet med denna avhandling är undersöka hur de amyloida peptiderna A β 1-40, A β 1-42 och IAPP påverkar pericyter i Alzheimers sjukdom. Studierna innefattar analyser av hjärnvävnad och näthinna från personer med AD och från kontrollpersoner, samt cellkulturer med humana pericyter som exponerats för olika aggregationsformer av de tre undersökta amyloida peptiderna. I vår första studie undersökte vi om olika aggregationsformer av A β 1-40 och A β 1-42 påverkar pericyter på olika sätt, samt om dessa amyloida peptider kan vara involverade i den pericytförlust som tidigare rapporterats i hjärnan vid AD. Våra resultat, i linje med tidigare studier, visade färre pericyter i hjärnan hos personer med AD jämfört med kontrollpersoner. Vi fann även att förändringar i pericytpopulationen i hjärnan hos personer med AD framförallt är kopplat till A β 1-40, och inte i lika stor utsträckning till A β 1-42. Våra cellkulturstudier visade vidare att den fibrillära formen av A β 1-40 relativt snabbt inducerade pericytdöd, medan dess oligomera och monomera former inte har samma starka effekt. Monomer A β 1-40 visade sig dessutom påverka pericyters celldelning positivt, något de andra två aggregationsformerna saknade. Både oligomer och fibrill A β 1-42 minskade överlevnaden av pericyter, men endast efter en längre exponeringstid.

Trots att studie I visade att A β 1-42 inte är lika toxisk för pericyter som A β 1-40, ville vi i studie II undersöka om A β 1-42 påverkar pericyter på något annat sätt. Med hjälp av cellkulturstudier visade vi att A β 1-42, på ett aggregationsberoende sätt, påverkar pericyters klyvning och frisättning av lösligt NG2. NG2 är en proteoglykan som är avgörande för pericyters funktion, men dess lösliga form är dessutom viktig för nybildning av kärl och minnesinlagring, vilket gör NG2 intressant ur ett AD perspektiv. Våra studier visade även att den A β 1-42 inducerade klyvningen av NG2 medieras via matrixmetalloprotein 9.

I studie III ville vi undersöka hur IAPP påverkar pericyter i hjärnan. Med hjälp av hjärnvävnadstudier visade vi att både personer med AD och kontrollpersoner hade ett litet antal pericyter innehållande IAPP-inklusioner. Dessa pericyter uppvisade även cellkärnsförändringar, vilket indikerar irreversibel cellskada. Antalet pericyter

med IAPP-inklusioner skiljde sig inte åt mellan AD och kontroller, men personer med T2D hade dubbelt så många. Att IAPP påverkar pericyter negativt bekräftades i våra cellkulturstudier, där vi kunde se att pericyterna tog upp oligomer IAPP och försökte bryta ner peptiden genom försvarsmekanismen autofagi. Cellens nedbrytningsförmåga var dock försämrad och IAPP började istället ansamlas intracellulärt. Ansamling av IAPP var vidare kopplat till nedreglering av skyddande NG2 och ökad pericytdöd.

I studie IV har vi, för första gången, visat att IAPP kan ansamlas i ögats näthinna och att dess pericyter också är påverkade vid AD. Vi fann även en koppling mellan nivåer av biologiskt aktivt IAPP och antalet retinala pericyter i AD patienter. Nivåer av IAPP och pericytantal i retina korrelerade dessutom med samma variabler i hippocampus, vilket indikerar att retina kan användas för att studera patologiska förändringar i hjärnan.

Sammanfattningsvis visar den här avhandlingen att pericyter, både i hjärna och retina, är påverkade vid AD. Resultaten föreslår även species- och aggregationsberoende skillnader i effekt mellan olika amyloida peptider på populationen av pericyter. Detta är helt ny information som är viktig att ta i beaktning vid vidare forskning på nya diagnostiska verktyg och behandlingsalternativ för AD och andra sjukdomar med vaskulära komplikationer såsom T2D.

1. Background

1.1 Alzheimer's disease

Alzheimer's disease (AD), named after the German neuropathologist Alois Alzheimer, was first described already in 1906 (1). Today it is considered the most common form of dementia, with approximately 47 million individuals affected worldwide in 2017 (2). The cost of caring for AD patients is an economical burden for the society and it will only increase as the number of patients continues to grow due to longer life expectancy. Despite extensive research over more than a century, there are as of today still no FDA approved medical drugs that can inhibit or cure AD. Only drugs for symptom relief are available to patients suffering from this cruel disorder.

1.1.1 Clinical symptoms

Approximately half of the population over 65 years of age have experienced some forgetfulness, such as an inability to remember the names of familiar people or where they have put things. This benign decline in memory and other cognitive functions is considered to be a part of normal ageing (3). However, when the forgetfulness affects activities of daily life, potential development of dementia should be suspected. The most characteristic clinical symptom of AD is a progressive decline in short-term memory that affects daily life and, for those who are still working, job performance. The affected person experiences that it is harder to learn new skills and to concentrate. As the disease progresses, the ability to perform ordinary tasks such as taking care of finances or preparing dinner, diminishes. It is often around this time that it becomes more obvious to family, friends and relatives that something is wrong. Along with the cognitive deterioration, these patients also commonly exhibit emotional disturbances with symptoms such as depression, apathy, anxiety, agitation and other psychiatric problems manifesting themselves (4).

1.1.2 Neuropathological manifestations

The clinical symptoms seen in AD patients are due to characteristic alterations in the brain, starting as much as 15-20 years before any clinical symptoms appear (5, 6). The first striking finding is the shrinkage of the brain (see Figure 1). This atrophy can be seen in earlier stages of the disorder in brain regions such as hippocampus and entorhinal cortex; regions important for memory formation (7, 8). As the disorder progresses, this dramatic atrophy can also be found in the temporal, parietal and frontal lobes as well (reviewed in (9)). On a more microscopic level, changes associated with the disease are the presence of intracellular neurofibrillary tangles (NFTs) and senile plaques (10-13). NFTs can be seen in immunohistological stainings of the brain as neuronal tangles. These tangles are composed of aggregated neuronal hyperphosphorylated tau, a microtubule-associated protein important for stabilizing neurons (Figure 2A). The NFTs in preclinical and early stages of the disorder are seen in the medial temporal lobe (hippocampus and entorhinal cortex) and are then spread, following to a well described pattern, to the associative neocortex (10, 14). This tau spreading pattern can be divided into 6 stages, called Braak stages, and is used in neuropathology to determine the level of severity. The tau spreading can also be visualized using ^{18}F -FDG-PET/CT where PET imaging tracers can help show tau depositions in the brain (15). The accumulations of aggregated hyper-phosphorylated tau are believed to underlie the loss of synapses and the degeneration of neurons seen in the AD brain.

Senile plaques are mainly composed of extracellular depositions of the peptide called amyloid beta ($\text{A}\beta$) (therefore also called $\text{A}\beta$ plaques), which can be visualized using immunohistological stainings of the AD brain (Figure 2B). These plaques arise, according to the most widely accepted idea “the amyloid cascade hypothesis”, due to an accumulation of $\text{A}\beta$. The accumulation is believed to be caused in turn by an imbalance between production and clearance of the peptide (16). The formation of toxic depositions leads to the neuronal dysfunction, synapse loss, tau hyperphosphorylation and cell death seen in the AD brain (17, 18). The plaques can appear in various forms described as diffuse or dense-cored plaques based on their morphological phenotype. In preclinical and early stages of the disease, $\text{A}\beta$ plaques are primarily found in the neocortex but as the disease progresses, they spread to allocortical brain regions, the striatum and also, in end stages, to the cerebellum (13), a spreading pattern that can be staged according to the CERAD/ABC staging and Thal stages (13). The amount and the spread of $\text{A}\beta$ in the brain can, just like tau pathology, be monitored in patients using ^{18}F -FDG-PET/CT and $\text{A}\beta$ PET ligands (19).

The accumulation of $\text{A}\beta$ and tau is often accompanied by neuro-inflammation and activation of astrocytes and microglial cells (Figure 2C). These activated glial cells are frequently found in close association with senile plaques, where also increased

expression of markers associated with inflammation, such as various chemokines, cytokines and cell specific markers, are found (20, 21). The general idea is that the neuro-inflammation and gliosis in the AD brain is a response to the cell death and atrophy associated with accumulation of A β and tau pathology, but it has also been suggested that the inflammatory processes act as a trigger, with cytokines and chemokines induce a vicious circle fuelling AD pathology (22).

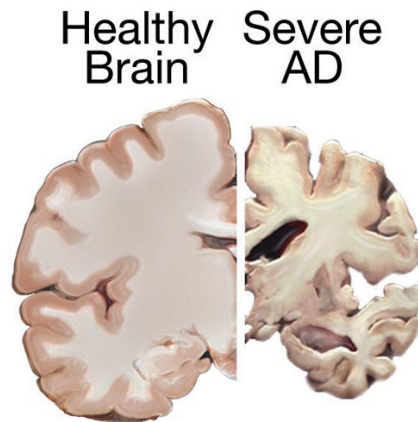


Figure 1.

Images demonstrate the shrinkage of the brain associated with Alzheimer's disease (AD). To the left, a healthy brain and to the right an AD brain displaying severe atrophy. National Institutes of Health. Alzheimers_brain.jpg [image] https://commons.wikimedia.org/wiki/File:Alzheimers_brain.jpg (CC0) <https://creativecommons.org/publicdomain/zero/1.0/>

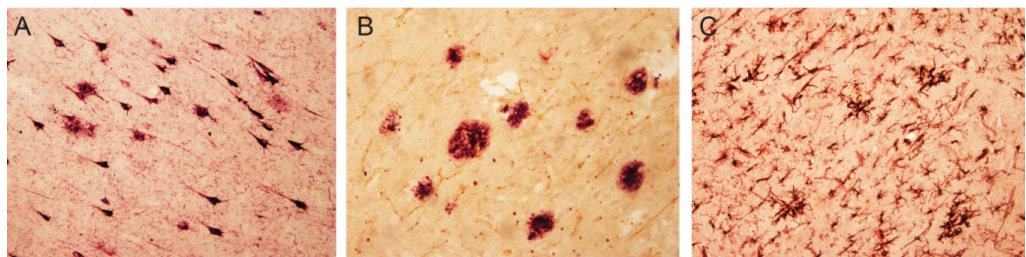


Figure 2.

Images demonstrate the characteristic hallmarks for Alzheimer's disease (AD); neurofibrillary tangles (NFTs)(A), senile amyloid beta (A β) plaques (B) and reactive microglial cells, indicative of neuroinflammation (C).

1.1.3 Vasculopathy in AD

In addition to the hallmarks mentioned above, the majority of AD patients also display pathological vascular changes. Several studies have shown impaired blood brain barrier (BBB) function. For example, reduced expression of the tight junction protein Zonula Occludens-1 (ZO-1) has been seen in AD patients where the reduction was associated with a compromised BBB and vessel integrity as well as increased leakage of the blood-borne proteins (23). Further evidence for a BBB leakage in AD patients has been demonstrated in studies showing increased infiltration of fibrinogen into the brain parenchyma (24). Moreover, an increased influx of albumin from blood to brain, measured by analysing the ratio between plasma albumin levels and CSF albumin levels (Q-albumin) (25-30), has repeatedly been reported to occur in AD patients.

Apart from BBB dysfunction, other vascular changes in AD patients have also been described including impaired vessel function (31), thickening of vessel basement membranes (31), and aberrant angiogenesis (32). Additionally, more than 80% of AD patients display cerebral amyloid angiopathy (CAA), a phenomenon characterized by A β depositions in cortical and leptomeningeal vessel walls (CAA-type 1) and cortical/leptomeningeal vessel walls (with the exception of cortical capillaries) (CAA-type 2) (33-35). This phenomenon together with the other reported vascular abnormalities are considered to be associated with higher risk of the reduced blood flow (36), micro bleeds (ruptures of small vessels) (37-39) and intracerebral haemorrhage (40) occasionally seen in these patients.

1.1.4 Current diagnostics

In Sweden, the diagnostic process starts with an initial consultation with a physician at a primary care centre, who will examine and evaluate the need for a cognitive investigation. If a neurocognitive disorder is suspected, a basic cognitive investigation is performed at the care centre. The investigation includes neurological and psychiatric evaluation, measurement of blood pressure, drawing of blood samples and the patient is also referred for a computed tomography (CT) of the brain. In addition, an occupational therapist tests memory and other cognitive functions such as spatial awareness, attention and language using various cognition screening tests such as the MMSE (41) or MOCA (42) and the clock test (43). These procedures aim to differentiate patients with various neurocognitive disorders/dementia diagnoses and also to exclude those with hypothyroidism, hypercalcemia or brain tumour, diagnoses that can be associated with cognitive symptoms but do not primarily cause neurodegeneration (44). In cases where the basic investigation fails to confirm a diagnosis, the patient is referred to the specialist clinic for more refined investigations including magnetic resonance

imaging (MRI) of the brain, neuropsychological evaluation, positron emission tomography (PET) scan and lumbar puncture for CSF analysis. CSF is analysed in order to study alterations in the core biomarkers A β , phosphorylated tau (p-tau) and total tau (t-tau) (45), as altered levels of these CSF biomarkers have been shown to mirror the tau and A β pathology in the AD brain.

1.1.5 Need for new diagnostic tools

The extensive research within the field of AD has produced along several promising new approaches now being tested in ongoing clinical trials. However, given that the neuropathological changes associated with AD start already 15-20 years before clinical symptoms are manifested (5, 6) and since AD patients are a heterogeneous group, in whom many different pathological pathways may lead to similar cognitive symptoms, it is important to find biomarkers and diagnostic tools that can identify AD pathology at the very earliest stage of its progression. One approach is to find new blood and CSF biomarkers associated with pathological changes characteristic of the disorder. Inflammatory signal substances in CSF such as cytokine IL-6 (46) and YKL-40 (47, 48) are being investigated; the former is associated with cognitive decline (49) and it has been suggested that the latter is an early AD marker (47). Other examples of new promising CSF biomarkers are markers indicative of synapse breakdown/neuronal injury, for example neurogranin (50), neurofilament light chain (NFL) (51, 52) and matrix metalloproteinase-9 (MMP-9) (53). Markers related to the pathological vascular changes seen in AD, for example vascular cell adhesion molecule-1 (VCAM-1) (54), vascular endothelial growth factor (VEGF) (55) and hyaluronic acid (56, 57) have also been studied.

Another new and interesting approach is to examine the retina of the eye in order to study changes occurring in the brain. As opposed to the brain, the eye is more easily accessible and much research has focused on retinal imaging as a potential non-invasive method for diagnosing AD. Indeed, studies have demonstrated retinal A β depositions that correlate with AD severity (58-60) and retinal vascular alterations correlating with cerebral A β plaque load in AD patients (61). These findings encourage further research on retinal imaging as a promising diagnostic tool.

1.1.6 Familial types of AD

Inherited forms of AD, called familial types of AD (fAD), account for 1-3 % of all AD cases and can often be traced back several generations with at least one parent diagnosed with fAD. The onset of fAD occurs early in life, around 30-40 years of age, and the underlying cause of the disease is one or several known gene mutation(s). A couple of different known mutations can cause fAD, the most common being mutations in PSEN1 and PSEN2 genes, encoding presenilin 1 (PS1)

and presenilin 2 (PS2), subunits of γ -secretase, as well as mutations in the A β precursor protein (APP) sequence (62). These mutations all lead to altered processing of APP and increased production of A β (63, 64).

1.1.7 Sporadic type of AD

In the majority (around 95-97%) of all AD cases occurrence is sporadic, i.e. without any known genetic causes. The greatest risk of developing sporadic AD is age and the onset in a majority of cases occurs at 65 years or older. After 65 years of age, the risk of developing AD doubles every fifth year and by the age of 80, one in three people suffers from the disorder. In addition to the inevitable risk factor of getting older, there are indications that modifiable lifestyle risk factors such as smoking (65), diet (66, 67), inadequate physical activity (68, 69), reduced cognitive stimulation as well as social interaction (70) may increase the risk of developing AD. Another factor known to play a role in AD progression is education, where a higher level of education can be seen as protective as studies have demonstrated that higher levels of education delay the onset of AD (71, 72). Studies also indicate that other factors, such as inflammation has been indicated to increase the risk of AD and studies have demonstrated elevated levels of inflammatory markers both in the AD brain (73, 74) and in the blood of AD patients (75). Furthermore, epidemiological studies show that treatment with certain nonsteroidal anti-inflammatory drugs (NSAID) reduces the risk of developing AD, delays the onset, slows the progression of the disease and reduces the severity of the cognitive symptoms (76-78).

The strongest genetic risk factor for sporadic AD is the lipoprotein transporter apolipoprotein E (ApoE), which exists in three forms, APOE- ϵ 2, APOE- ϵ 3, and APOE- ϵ 4. The APOE- ϵ 4, present in 10-15 % of the population, increases the risk of developing AD. Carriers with one allele of APOE- ϵ 4 have approximately a 2-3 times higher risk whereas carriers with two alleles of APOE- ϵ 4 have up to a 12 times higher risk of developing AD. The exact role of the lipid transporter ApoE in AD is still being debated, but it is believed to contribute to the disorder by affecting the accumulation as well as the removal of A β from the brain (79-81). Interestingly, the protective role of NSAID mentioned above was more pronounced in patients carrying one or two alleles of APOE- ϵ 4 (82).

Finally, several studies have also demonstrated a higher risk of AD in patients suffering from cardiovascular disorders such as stroke, myocardial infarction and atherosclerosis (83) as well as type 2 diabetes (T2D) (84-89). The latter in particular has been highlighted recently as studies have shown that T2D patients run twice the risk of developing AD (90, 91), and that the risk increases with an increasing number of vascular complications associated with T2D (such as diabetic foot and retinopathy) (92). Evidence for the involvement of vascular alterations is further

strengthened by the fact that the majority of AD patients, as described above, display multiple pathological vascular alterations. The high prevalence of AD patients with vascular changes contributes to an overlap between AD and vascular dementia diagnoses, where the latter dementia diagnosis is characterized by reduced blood flow caused by dysfunctional vessels (93).

1.2 Pericytes

The underlying cause of the vascular changes in AD is not completely understood, but several studies point to changes in the pericyte population as an important event implicated in AD pathology. These cells were first described in the late 19th century (94, 95) and were given their name by Zimmermann in 1923 (96) because of their location associated with the vessels (peri= around and cyte= cell) (Figure 3). Pericytes stabilize small vessels such as capillaries, precapillary arterioles and post-capillary venules (97, 98) and should be differentiated from smooth muscle cells that stabilize larger arteries. Pericytes can be found throughout the body, with the highest pericyte-endothelial cell ratio in the brain and in the retina (1:1) compared to other tissues (where it is closer to 1:10) (99-101).

1.2.1 Markers for pericyte identification

Rodent studies have demonstrated that pericytes express various markers depending on their developmental stage, origin, milieu and localisation along the vasculature. Consequently, there is not one antibody that can single-handedly capture all pericytes in the tissue. Therefore, when identifying pericytes, several markers are usually used in combination with analysis of their localisation along the vessel (reviewed in (102)). The most commonly used marker for pericyte identification has for many years been PDGFR- β , a transmembrane tyrosine kinase receptor that is important for regulating pericyte proliferation, cell growth, differentiation and development. Examples of other markers that have been used, with varying results, are laminin, which is enclosing pericytes on both sides (103), but also smooth muscle actin (α -SMA) (104-106), CD13 (107), NG2 (108), RGS5 (109), endosialin/TEM-1 (110), desmin (111), vimentin (112) and Kir 6.1 (113). However, although antibodies against pericyte markers such as PDGFR- β , yield good results when staining rodent tissue, antibodies are less effective or are not sufficient in human material. The fixation step has been shown to be essential for a successful staining of pericytes in human tissue. Unfortunately, most brain banks only offer tissue fixed using standard protocols (fresh frozen or formalin fixed/paraffin embedded), which often restrict the possibilities of obtaining a good staining with clear pericyte cell bodies.

1.2.2 Component in blood brain barrier

Pericytes play a crucial role in maintaining the blood-brain barrier (BBB). This barrier regulates the passage of nutrients, ions and oxygen from blood to brain and transports waste products and toxic substances from brain to blood (114). It is built up of endothelial cells, held together by tight junctions to form non-fenestrated vessels (115, 116), which constitute the physical barrier. However, interactions with stabilizing pericytes, astrocytic end feet, microglial and neurons are also of great importance for a healthy BBB (117). During embryogenesis, pericytes invade neural tissue together with endothelial cells (118) and the role of pericytes in induction of a functional BBB has been demonstrated in pericyte-deficient mice displaying a dysfunctional BBB (118). In particular, the expression of the pericyte marker PDGFR- β has been shown to be important for BBB formation as studies using *Pdgfr β* knockout mice demonstrate a reduced pericyte coverage leading to leaky vessels (119, 120). Furthermore, studies in mice show that detachment of stabilizing pericytes during embryonal stages leads to impaired BBB integrity and leaky vessels (121). However, the need for pericytes in maintaining a functional BBB is not limited to embryonal stages but continues throughout the lifespan (122). Pericytes in the adult brain regulate the BBB permeability by controlling the expression of endothelial tight junction proteins, forming a physical barrier between endothelial cells and pericytes (122). Pericytes also regulate levels of molecules inside the brain by taking up and degrading small molecules via pinocytosis, lysosomal degradation and endocytosis via specific receptors, such as low density lipoprotein receptor-related protein (LRP-1) and receptors for advanced glycation end products (RAGE) (123). Furthermore, together with smooth muscle cells, pericytes participate in the intramural periarterial drainage pathway (IPAD) (124). This drainage pathway allows molecules to be eliminated from the brain along the basement membranes of capillaries and arteries and the contracting property of pericytes/smooth muscles cells forces the direction of IPAD in the opposite direction to the blood flow (125).

Pericytes in the retina play the same role as brain pericytes in the case of regulating molecules crossing over from the blood. The barrier between the blood and the retina is called the blood-retina barrier (BRB) and consists of two parts; the outer BRB where tight junctions are formed between retinal pigment epithelial cells, and the inner BRB composed of tight junctions between retinal capillary endothelial cells covered by pericytes and glial cells (126, 127). The functions of the BRB are similar as those of the for BBB; control of fluids, nutrients, glucose and oxygen from crossing over, as well as preventing toxic molecules from entering the retina (128). However, in the case of BRB breakdown, these harmful substances will damage the retina with detrimental consequences. For example, a damaged outer BRB leads to degeneration of the retina, loss of visual function and, ultimately, blindness (128). Damage to the inner BRB also leads to various medical conditions, such as diabetic retinopathy, with blindness as a consequence (129, 130).

Interestingly, one of the first clinical symptoms of diabetic retinopathy is the loss of retinal pericytes (131).

1.2.3 Vessel stability and angiogenesis

Embedded within the basement membrane, pericytes with their multiple long processes are wrapped around endothelial cells (ECs), give support to the vessel they surround (132). Pericytes do not only support vessels already formed, but they also play a very important part as stabilizers when in the formation of new ones (133). The formation of new blood vessels, vasculogenesis, occurs primarily during the embryonal development but angiogenesis (formation of new vessels from pre-existing vessels) happens, albeit to a limited extent, throughout life (134). In later stages of the angiogenesis process, when the endothelial tube is formed, the sprouting endothelial cells secrete platelet-derived growth factor (PDGF), which binds to the PDGFR- β expressed by pericytes resulting in recruitment of stabilizing pericytes to the newly formed capillaries. Recruitment of pericytes is also crucial for regulating endothelial differentiation and growth arrest (135-137). Studies in mice show further the importance of pericyte stabilization, as mice with complete knockout of *Pdgfr β* or *Pdgfr β* demonstrate a lack of pericytes and already die at the embryonic stages due to microvascular haemorrhage (119, 120). If the PDGF- β /PDGFR- β signalling between ECs and pericytes does not work properly, pericytes are not recruited to the vessel, leading to uncontrolled ECs proliferation, unstable and consequently leaky vessels (138, 139).

1.2.4 Secretory properties

Chemokines and cytokines are signal molecules important in the cross-communication between cells as well as during angiogenesis/remodelling and inflammatory responses. Such signal molecules are secreted from pericytes in order to regulate the immune response, endothelial growth and differentiation (140). Some studies suggest that brain pericytes can acquire a macrophage/microglial like morphology and start to express microglial markers, enabling them to function more like a macrophage/microglial cells in the event of stroke or other potential harmful conditions (141). Whether these cells should be seen as pericytes or whether they should be counted as pericytes differentiated to macrophages/microglia, is still being debated. However, these cells secrete various factors (chemokines, cytokines, growth factors and components of the extracellular matrix) to their surroundings (reviewed in (142)). For example, pericytes secrete Angiopoietin-1 to promote remodelling, maturation and differentiation of endothelial cells (132, 143) and vascular endothelial growth factor (VEGF) to provide guidance for endothelial movement and tube formation during angiogenesis (144). Pericytes also produce

matrix metalloproteinases (MMPs) to degrade the extra cellular matrix (ECM) (145, 146). The production of pericyte MMP adds to the pool of endothelial MMP production that is secreted to loosen up the ECM, pave the way for the new vessels and detach pericytes from the vessels undergoing angiogenesis. Despite the important roles of MMPs in angiogenesis, these enzymes are also known to be key mediators during small-vessel disease in dementia (147) and stroke (148). Both in vitro studies (149) and in vivo (150) studies demonstrate that pericytes contribute to inflammation-induced BBB breakdown through the secretion of MMPs.

1.2.5 Neural/glial antigen 2

As described above, pericytes express and secrete various molecules that contribute to vascular integrity. One example of such a molecule is the transmembrane proteoglycan neural/glial antigen 2 (NG2), expressed by pericytes and oligodendrocyte precursor cells (OPCs). The surface-bound version of NG2 is important for pericyte survival, migration and proliferation as well as in interactions between pericytes and endothelial cells. Studies show reduced pericyte ensheathment of endothelial cells, diminished formation of endothelial cells, and decreased assembly of the vascular basal lamina in pericyte specific NG2-null mice (151). Moreover, reduced pericyte proliferation/migration, endothelial tight junction formation and pericyte-endothelial network formation have been seen in in vitro NG2 knockdown studies (151). The intracellular part of the membrane-bound NG2 has also been shown to protect OPCs from oxidative stress by binding to OMI/HtrA2, a mitochondria-derived protease that will initiate apoptosis as a response to oxidative stress (152).

Interestingly, NG2 can also be cleaved from the surface and the shed version (sNG2) appears to have other interesting functions. In vitro studies show that sNG2 promotes angiogenesis and the migration of endothelial cells via its binding to galactin-3 expressed on endothelial cells. In vivo studies on NG2-knockout mice also show that lack of soluble NG2 results in a marked reduction of N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) in pyramidal neurons (153). These findings, highlighting sNG2 as a crucial component in memory formation, thus make alterations in NG2 and sNG2 levels interesting from a dementia perspective. Indeed, an earlier study demonstrated reduced NG2 immunoactivity in OPCs in post-mortem brain tissue from AD patients (154). The same study demonstrates reduced levels of sNG2 in CSF from AD patients and a correlation between sNG2 and A β 1-42 in CSF of in these patients, suggesting that A β 1-42 affects the amount of sNG2. This was confirmed in cell culture studies where reduced NG2 levels were seen in both lysates and supernatants from cultured OPCs after exposure to fibril A β 1-42 (154).

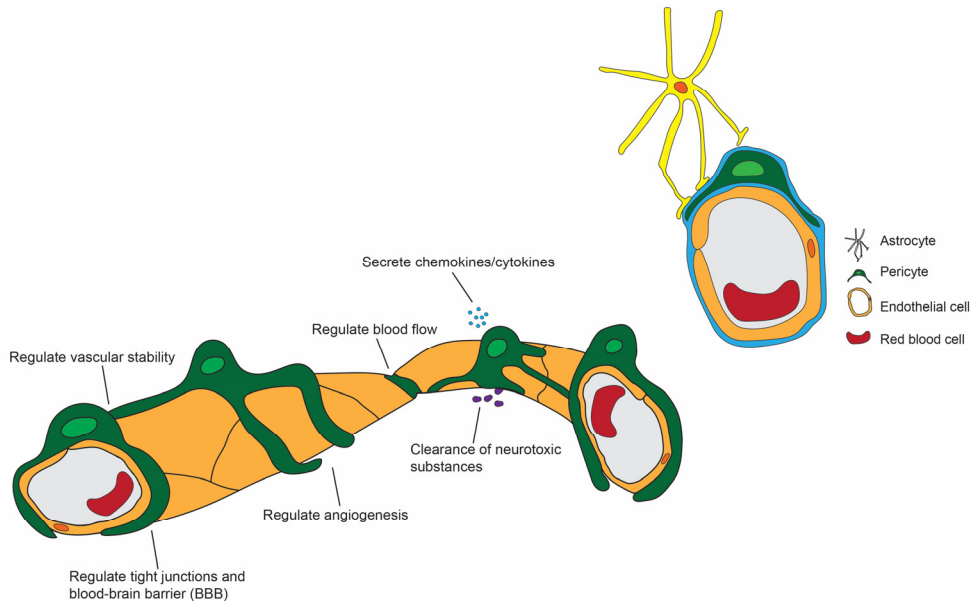


Figure 3. Schematic pictures of the different neurovascular components of brain capillaries and the main functions of brain pericytes. Endothelial cells (orange) are held together by tight-junctions to form non-fenestrated vessels and are surrounded by pericytes (green). Pericytes are ensheathed in the basal lamina (blue) and interact with astrocytic end-feet (yellow).

1.2.6 Pericytes in AD

Given the vital role of pericytes in the maintenance of vessel integrity and function, it is plausible that alterations in the pericyte population underlie some of the vasculopathy seen in AD patients. Indeed, studies have demonstrated a 60% reduction in pericyte numbers (25) and 30% less coverage of PDGFR- β positive pericytes on brain capillaries in AD patients compared to controls (25, 28). This pericyte loss correlated with increased A β plaque load (25). Other signs of pericyte degeneration have also been found in AD patients, including pinocytotic vesicles, mitochondrial damage and large lipid granules (31, 155). Pericytes with apoptotic features have also been associated with A β depositions in the vessel wall, i.e. CAA (33), and studies suggest that the CAA successively replaces mural cells in the basement membranes (156). This replacement results in a loss of contractile properties, impairing the IPAD-mediated A β clearance leading to increased accumulation of the A β peptide. This hypothesis is supported by studies showing that reduced contractile ability in aged mice impairs the drainage of peripherally administered human A β , which in turn enhances cerebral accumulation of the peptide (157). Further implication of pericytes loss for AD pathology has been shown in a study using pericyte deficient mice overexpressing APP. The study

shows that the deficiency of pericytes initiates AD-like changes (158) such as increased A β levels, A β plaque load and neurodegeneration. It was proposed that these changes occur due to reduced clearance of the A β from the brain as these transgenic mice moreover showed reduced levels of the A β clearing receptor LRP-1 (158).

1.3 Amyloid beta

The well-documented presence of A β plaques in AD highlights A β as one of the most important actors in AD progression. This peptide accumulates, due to an imbalance between production and clearance of A β (16), and aggregates into toxic depositions. These depositions in turn are believed to underlie the pathological changes associated with AD, including neuronal dysfunction, synapse loss and cell death.

1.3.1 APP cleavage and the formation of A β

The A β peptide is the cleavage product yielded after proteolytic cleavage of the amyloid β precursor protein (APP), encoded on chromosome 21. The transmembrane protein APP is expressed by various cell types both in the periphery and in the central nervous system (CNS). The membrane-bound form of APP is believed to play an important role in CNS development as *in vivo* studies demonstrate involvement of APP in neuronal migration in early embryogenesis in mice (159) as well as in synapse formation and maintenance in rats (160). Furthermore, APP knock-out mice display reduced brain mass (161), altered long-term potentiation (LTP) (162) and fewer dendritic spines (163), indicating that APP has a role in establishing the neuronal network. The APP can be cleaved from the membrane by various enzymes, resulting in different end products. If APP is cleaved by α - and γ -secretases, non-amyloidogenic molecules such as sAPP α , and C-terminal fragments CTF 83 and AICD (see Figure 4) are formed. The exact functions of sAPP α are not fully understood but previous studies show that it has a protective role for cultured neurons (164, 165) and enhanced learning and memory have been seen in rodents after intracerebroventricular administration of sAPP α (166, 167). If APP instead is being cleaved by β -secretase (BACE-1) and γ -secretases, the result is the formation of the amyloidogenic amyloid beta (A β) peptide (168, 169). Different species of the peptide are formed dependent of the cleavage sites of the γ -secretases, where two of the most abundant forms are A β 1-40 and the two amino acid residues longer A β 1-42.

Non-amyloidogenic pathway

Amyloidogenic pathway

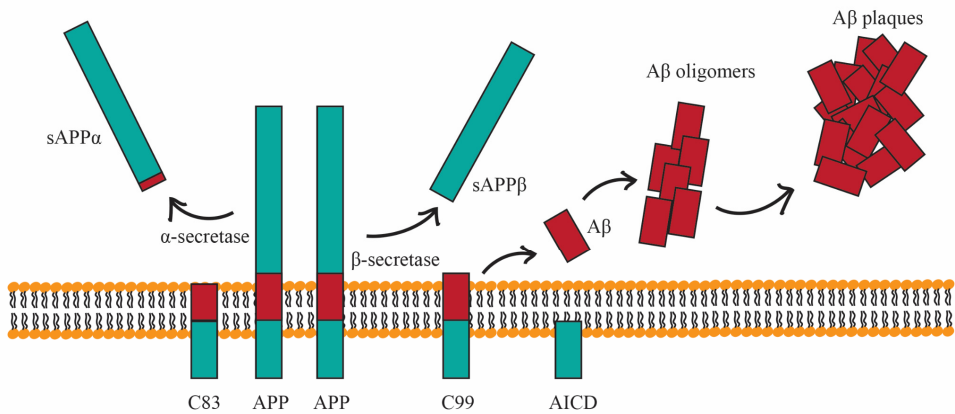


Figure 4.

Cleavage of the transmembrane protein amyloid precursor protein (APP). APP can be cleaved via two different pathways: amyloidogenic pathway and non-amyloidogenic pathway. Sequential cleavage of APP by α -secretase and γ -secretase generates sAPP α and C83 whereas sequential cleavage of APP by β -secretase and γ -secretase results in sAPP β , C99, AICD and A β . The A β peptide can aggregate and form soluble oligomers as well as insoluble A β fibrils, primarily consisting of A β fibrils.

1.3.2 A β 1-42

The A β peptide is crucial for maintaining function in the healthy brain. Studies indicate the importance of A β in memory formation, as A β 1-42 (in picomolar concentrations) enhances LTP and hippocampal-dependent memory (170) and impaired synaptic plasticity and memory are seen in BACE-1 knockout mice (171). In vitro studies using a mouse neuronal stem cell line, also demonstrate that A β 1-42 plays a role in the formation of new glial cells (172). Furthermore, in the absence of growth factors, the monomeric form of A β 1-42 has been shown to possess neuroprotective features on cultured neurons (173). The A β 1-42 peptide is however very prone to aggregate, leading to the formation of soluble A β 1-42 oligomers, protofibrils as well as insoluble fibrils, the latter being the major component of senile plaques (174). The distinction between protofibrils and fibrils is in contention today but usually fibrils are defined as thin (12-15nm), elongated (100-1000 nm) and branched structures consisting of β -sheets whereas protofibrils are defined as the first elongated unit appearing in fibril formation (175). Although, both oligomers, protofibrils and fibrils of A β 1-42 have all been shown to evoke inflammatory responses (176), a growing number of cell culture studies indicate that it is the A β oligomers that are the most toxic variant and thus underlie the neuronal cell death seen in AD patients (177).

1.3.3 A β 1-40

The majority (around 90%) of the A β peptides produced in the CNS is comprised of the shorter version A β 1-40 peptide (178). This peptide is considered to be “the good sibling to A β 1-42” as in vitro studies suggest that A β 1-40 is beneficial and neuroprotective. For example, both proliferation and neurogenesis of cultured neuronal stem cells have been shown to be promoted by A β 1-40 (172). Supplementation of A β 1-40 has been shown to rescue cultured neurons from dying of inhibition of their endogenous A β production (179). However, even though A β 1-40 is not as prone to aggregate as A β 1-42, it can still deposit and aggregated A β 1-40 is found particularly in CAA.

1.3.4 Amyloid peptides and pericytes

How pericytes are affected by A β has hardly been investigated, but a limited number of studies show a link between A β and pericyte death. Reduced survival of cultured human primary brain pericytes has been seen after prolonged exposure to A β 1-42 (180, 181), but not to A β 1-40 (181, 182). Interestingly, Dutch-type A β 1-40, an A β 1-40 version containing an aggregation-promoting mutation (181, 182), has however also been shown to induce pericyte death, suggesting that an aggregated form of this A β species is particularly toxic to pericytes. The impact of A β on pericytes has been shown further in in vivo studies, where cerebral A β plaque load in mice overexpressing APP correlates with reduced number of pericytes (183). In addition, studies using ex vivo rodent brain slices show contraction of capillaries at the location of pericytes after exposure to A β 1-42 oligomers (184). These findings were verified in human studies demonstrating reduced diameter of capillaries from human subjects with A β deposition compared to subjects without such depositions (184). These first steps to understanding how A β induces pericyte loss in AD need to be followed up by studies investigating the underlying signalling pathways and mechanisms involved.

1.4 Islet amyloid polypeptide (IAPP)

The hormone peptide Islet amyloid polypeptide (IAPP) or amylin is primarily associated with T2D as depositions of this peptide are found in the pancreas, where it is believed to underlie the β -cell death seen in these patients (185). However, in recent years IAPP has also been associated with AD as inclusions of IAPP have been found in the brain of demented T2D patients and AD patients (186, 187).

1.4.1 Kinetics

The general consensus is that IAPP is primarily expressed by the β -cells of the pancreatic islets of Langerhans (188, 189). Some studies also report IAPP mRNA in human brain regions such as the hypothalamus and arcuate nucleus (190-192). However, these mRNA levels are very low compared to levels in the pancreas. In addition, similar amounts of human IAPP mRNA have been found in rat brain when using primers for the human IAPP gene indicating that these levels are background (186, 193). The IAPP peptide is synthesized as an 89-residue long peptide that undergoes several cleavage steps before the final 37-amino acid long and mature peptide is generated. Two modification steps, the addition of a C-terminal amine group and the addition of a disulphide bond between cysteine 2 and cysteine 7, are required in order for IAPP to be biologically active (194). When the mature IAPP is formed, it is packed tight together with a crystalline structure of insulin in β -cells granules (195). In the granules, insulin together with zinc, constitutes the granule core, whereas IAPP is localized in the halo (196). These two peptides are then co-secreted from the same granule in response to increasing glucose levels in the blood (197).

1.4.2 Physiological roles of biologically active IAPP

After a meal, blood glucose levels increase and in order for the cells in the body to take up the energy, insulin is released from the pancreas to facilitate the uptake of glucose from the blood. As IAPP is secreted together with insulin, a rise in IAPP accompanies the increased insulin levels. This IAPP surge regulates the amount of glucose being released from the stomach into the bloodstream and thereby helps to keep the glucose at a steady level (198). IAPP also regulates the post-meal glucagon secretion from islet α -cells (199) and after crossing the BBB it regulates the appetite by activating noradrenergic neurons in regions such as nucleus accumbens, dorsal raphe and area postrema (191, 200, 201).

1.4.3 Aggregation and pathological roles of IAPP

Although IAPP has many important physiological roles, it is evident that it can also play a pathological role. When the IAPP is secreted in high concentrations or if the peptide becomes modified in some way, it not only loses its biological activity but the peptide becomes unstable and starts to aggregate into toxic depositions. This is seen particularly in pre-diabetic stages where high levels of insulin and IAPP are secreted as a response to prolonged hyperglycaemia. It is not only the high concentrations of secreted IAPP, however, that initiate the aggregation process of IAPP, the lack of factors keeping the IAPP in its monomeric form also contributes. How IAPP is protected from forming aggregates within the granules is not fully

understood but studies suggest involvement of various components, such as insulin, in the complex granules. Under normal conditions, the insulin to IAPP ratio is 1:100 but in pre-diabetic stages when levels of insulin (and thereby also IAPP) are high, this ratio shifts to 1:20 (197, 202, 203). Insulin is no longer able to keep IAPP in its monomeric form and it starts to aggregate and form deposits. These deposits are found in the pancreas (185) in the majority of T2D patients, but also in other peripheral organs such as heart tissue (204) and kidneys (205).

Modification of IAPP, such as C-terminal deamidation and reduction of the C2-C7 disulphide bond, is another event that destabilizes the peptide making it more prone to aggregate. Interestingly, alterations in the amino acid sequence have been shown to be involved in the IAPP aggregation process as changes in specific aggregation-prone residue regions, such as the 20-29 residues as well as other regions, making IAPP unable to form amyloid fibrils (206-211). The fact that a proline-for serine substitution in position 28, as for example in rats, mice and pigs, also almost inhibits the formation of fibril IAPP seen in humans and cats (212). It is important to remember these structural differences in IAPP between species when studying the effect of IAPP in vivo and in vitro as the IAPP peptide might not act in the similar ways in other species and in humans. Moreover, similar to most amyloid peptides, including A β (213, 214), changes associated with environmental destabilisation, such as increased pH, altered concentrations of salt, oxidation, lipid oxidation and glycation have also been shown to change the normal conformational state of IAPP and thereby the stability and aggregation pattern of the peptide (194, 215-217).

1.4.4 The role of IAPP in AD

The role of IAPP in AD has been highlighted as deposits of this peptide have recently been found in the brain of demented type 2 diabetes (T2D) patients and patients with AD (186, 187). Interestingly, the IAPP peptide shares several features with A β , including β -sheet secondary structures (218), binding to the same receptors (219) and being degraded by the same protease, insulin-degrading enzyme (IDE) (220, 221). Earlier studies show that IAPP and A β co-localize in the human brain (186, 187) and in animal models, where IAPP has been injected, it has been demonstrated that A β and IAPP promote misfolding and aggregation of each other both in the brain and in the pancreas (187, 222). These in vivo findings are supported by in vitro studies providing evidence that IAPP and A β can seed each other (i.e. function as a template triggering each other's aggregation) (222-226) as well as form IAPP/A β oligomers (186). The significance of these findings is not fully understood as in vitro studies suggest that IAPP/A β complexes are less toxic than IAPP or A β oligomers alone (227). Furthermore, it has recently been suggested that IAPP plays a role in A β clearance, as studies in AD mice models show increased A β translocation from the brain into the blood after peripheral injections of IAPP or its

non-amyloidogenic analogue pramlintide (228). This protective role of IAPP in cognition is further demonstrated in preclinical studies, where transgenic AD mice improved cognitively after treatment with IAPP or pramlintide (228, 229). A clinical study, demonstrating a positive correlation between plasma levels of biologically active IAPP and cognition (230), supports the idea of biologically active IAPP being beneficial and further indicates that a loss of the same may be detrimental for the brain. Indeed, reduced levels of biologically active IAPP have been reported in plasma from patients with mild cognitive impairment (MCI) and AD patients (229, 231). Depositions of cerebral IAPP have also been seen in rats overexpressing human IAPP and interestingly, these rats also display neurological deficits (193). The exact role of IAPP in neurodegenerative diseases such as AD, has scarcely been investigated but studies show that IAPP monomers and oligomers circulate in the blood, cross the BBB, where they induce inflammation that consequently will lead to increased BBB permeability (201). Whether such an induction of BBB dysfunction is implicated in AD remains to be investigated.

1.4.5 IAPP and pericytes

It is not known how the vasculature and brain pericytes are affected by IAPP, but since IAPP has been shown to deposit in the wall of blood vessels and in perivascular spaces (186), it is tempting to speculate that this amyloid peptide also affects pericytes in a way that could potentially contribute to AD pathology. Whether pericytes express receptors binding IAPP is not shown, but since the amylin receptor 3 is found on most brain cells (including neurons, microglial cells and endothelial cells) in vitro (232, 233), it is possible that also pericytes express this receptor. A study demonstrating expression of one compartment of the heterodimerized amylin receptor (234), the calcitonin receptor (235), in rat pericytes, supports this idea. Studies also suggest that IAPP can bind to the RAGE receptor on β -cells (236) but whether IAPP also binds to RAGE and other A β -binding receptors, such as LRP-1, on pericytes remains to be investigated. Furthermore, it is not known whether pericytes could play a role in uptake and clearance of IAPP. However, in vivo studies demonstrating endothelial-dependent vessel relaxation in response to IAPP injections (237), do imply that pericytes, as regulators of capillary contraction, could be affected by IAPP. Given the increasing number of studies highlighting IAPP in AD pathology, a disorder accompanied by vasculopathy, it is crucial to investigate how the peptide influences and affects the vasculature and pericytes.

2. Aims of the thesis

The overall aim of this thesis is to investigate how amyloid peptides such as amyloid beta and IAPP affect pericytes in Alzheimer's disease.

The specific aims are:

- Investigating how A β 1-40 and A β 1-42 influence the hippocampal pericyte population ex vivo and in vitro (**Paper I**).
- Understanding how A β 1-42 impacts the pericyte proteoglycan NG2 and revealing the underlying mechanisms (**Paper II**).
- Examining how IAPP affects hippocampal pericytes ex vivo and in vitro (**Paper III**).
- Investigating retinal IAPP and its potential correlation with pericyte/vascular changes and hippocampal IAPP levels (**Paper IV**).

3. Material and methods

3.1 Human post-mortem tissue samples

3.1.1 Human post-mortem brain and eye tissue

Access to human midlevel hippocampus entorhinal cortex tissue and eye tissue from non-demented controls (NCs) and AD patients was gained through a collaboration with the Netherlands Brain Bank (NBB). The procedures of brain tissue and eye tissue collection were approved by medical ethical evaluation committee at the VU medical centre, Amsterdam and the studies were approved by the regional ethical board in Lund. The approval for the use of brain and eye tissue and clinical data for research purposes was obtained from all patients or their next kin in accordance with the International Declaration of Helsinki. Brain tissue from individuals included in two different cohorts were used; the first cohort (**Paper III**) consisted of tissue dissected at autopsy within 7 hours post-mortem, post-fixed in 4% paraformaldehyde (PFA) 14-20 hours and left in 30 % sucrose-PBS solution for 4 days. The tissue was then cut into 40 μ m thick sections using a cryostat and stored free floating with antifreeze cryoprotectant in -20 °C until analysis. The brain tissue from individuals included in the second cohort (**Papers I and IV**) consisted of fresh frozen hippocampus that were divided into two 3 mm thick sections; one for immunohistochemical stainings and one was homogenized for analysis of A β and IAPP levels. For immunohistochemical stainings, the frozen tissue was fixed in 4% PFA for 4 hours and left in 30% sucrose-PBS until they sank. The tissue was then sectioned into 40 μ m thick sections and stored free floating in antifreeze cryoprotectant at -20 °C until analysis. Frozen samples of human retina (right eyeball) (**Paper IV**) were either homogenized for IAPP analysis or cut in 1.0 x 0.5 cm pieces 0.5 cm from the optic nerve in the far peripheral superior part of the eye before fixed in 4 % PFA for 4 hours for immunohistochemical stainings.

3.1.2 Homogenization of human brain and eye tissue

Frozen samples of human retina (1.0 x 0.5 cm) and frozen hippocampal tissue (approximately 30 mg) (**Paper IV**) were homogenized by incubating the tissue in 100% formic acid (FA) overnight (ON) at room temperature (RT), centrifuged

13.000 x g for 20 min 4°C. The supernatant was thereafter collected, lyophilized and re-dissolved in DMSO to generate the S_{L-FA} fraction. The S_{U-FA} , P_{U-FA} and P_{U-FA-G} fractions of retina and hippocampus were generated by homogenizing the tissue in cell lysis buffer (Sigma-Aldrich) together with protease inhibitors at 4°C, ultra-centrifuged 350.000 x g for 1 hour at 4°C. The supernatant was collected and hereafter referred to as S_{U-FA} , the pellet was re-homogenized in FA (100%), incubated 1 hour at RT, centrifuged 13.000 x g for 20 minutes at 4°C before lyophilized and re-dissolved in DMSO, to generate the P_{U-FA} fractions. The P_{U-FA} fraction was further incubated in GuHCl (3M) for 2 hours at RT to generate the P_{U-FA-G} fraction.

3.1.3 Immunohistochemical (IHC) procedures

Immunohistological stainings of human post-mortem tissue against NG2 and laminin $\alpha 5$ (**Papers I and IV**) (Table 1), against IAPP and A β plaques (**Paper III**) (Table 1) were carried out by quenching the brain sections in H₂O₂ (3%) and methanol (10%) in KPBS for 30 min before they were put in Impress reagent kit blocking solution (BS) (Vector Laboratories) for 1 hour RT. The sections were then incubated with primary antibody in BS in 4°C ON and then the appropriate Ig Impress reagent kit secondary antibody (Vector Laboratories) for 2 hours RT, followed by peroxidase detection for 2 min (0.25 mg/ml diaminobenzidine and 0.012% H₂O₂).

3.1.4 Immunofluorescence (IF) procedures

Human post-mortem hippocampal tissue (**Paper III**) and retinal tissue (**Paper IV**) was immunofluorescent-stained by 1 hour incubation with blocking solution (BS) containing 5% serum from the source species of the secondary antibody in KPBS. The tissue was then incubated with primary antibodies (Table 1) in BS ON at 4°C. The primary antibodies were followed by incubation with appropriate fluorescence labelled secondary antibodies in BS RT for 1 hour in the dark. Finally, in order to reduce the auto-fluorescence in the staining, the tissue was treated with Sudan Black and then mounted with Vectashield Set mounting medium containing DAPI (Vector Laboratories).

Table 1.
Primary antibodies

Antibody	Specie	Dilution	Distributor	Used in paper
NG2 (MAB2029)	Mouse	1:200	Millipore	I-IV
NG2 (B5)	Mouse	1:200	Gift Bill Stallcup	II
PDGFR- β	Rabbit	1:50	RnD Systems	II
α -SMA	Mouse	1:200	Sigma-Aldrich	II
IAPP (T4149)	Rabbit	1:100	Peninsula Laboratories	III
IAPP (T4157)	Rabbit	1:100	Peninsula Laboratories	III
A β (6E10)	Mouse	1:5000	Covance	II, III
Laminin- α 5	Mouse	1:100	Dako	I, III
CD163	Mouse	1:100	Serotec	III
A133	Rabbit	1:1000	Gift Gunilla Westermark	IV
Ki67	Rabbit	1:200	Abcam	I
ICAM-1	Rabbit	1:200	RnD Systems	III
CD34	Rabbit	1:200	Abcam	III
A β (AS08 357)	Rabbit	1:1000	Agrisera	I

Table 2.
Secondary antibodies

Antibody	Specie	Dilution	Distributor	Used in paper
Alexa 488	Rabbit	1:500	Molecular Probes	II
Alexa 488	Mouse	1:500	Invitrogen	III
Alexa 594	Rabbit	1:500	Life Technologies	III
Cy3	Mouse	1:500	Jackson ImmunoResearch	II, III
Biotin conjugated	Rabbit	1:500	Dako	III
Dylight 549	Rabbit	1:500	Vector Laboratories	I, III
Impress	Mouse	*	Vector Laboratories	I, III
Impress	Rabbit	*	Vector Laboratories	III
HRP conjugated	Goat	1:20000	Abcam	IV
HRP conjugated	Goat	1:1000	Santa Cruz Biotechnology	IV

* Ready-made solution

3.1.5 Analysis of A β 1-40 and A β 1-42

Three hippocampal sections of each individual included in the study (**Paper I**) were placed in FA (100%) (10 μ l/mg) ON at RT. The solution was centrifuged at 13,000 x g for 20 min at 4°C, the supernatant was lyophilized and re-dissolved in dimethyl sulfoxide (DMSO). MesoScale Discovery V-Plex A β Peptide Panel 1 kit with electrochemiluminescence (ECL) detection technology was used according to manufacturer's instructions to analyse levels of A β 1-40 and A β 1-42. The ECL signal was quantified using MesoScale Discovery SECTOR Imager 6000.

3.1.6 Analysis of unmodified IAPP

Levels of unmodified IAPP in the S_{L-FA}, S_{U-FA} and P_{U-FA} fractions (**Paper IV**) were analysed using Human Amylin ELISA kit (Millipore) according to manufacturer's instructions. The fluorescence was measured at 355/460 nm using Biotek EON Microplate Reader.

3.1.7 Analysis of total (unmodified and modified) IAPP

Levels of total IAPP in the P_{U-FA-G} fraction (**Paper IV**) were analysed in an in-house developed direct ELISA with A133 (kind gift Professor Gunilla Westermarck) as detection antibody. Samples and positive/negative controls were allowed to adhere ON at 4°C in a clear 96-wells. The wells were then incubated with BS (1% BSA in PBS) 1 hour at RT, before incubated with A133 antibody 2 hours at RT and goat anti-rabbit HRP secondary antibody (Santa Cruz Biotechnology) 1 hour at RT. TMB substrate solution was used to detect HRP activity and the absorbance was measured at 450/540 nm using Biotek EON Microplate Reader.

3.1.8 Dot blot analysis of IAPP levels

Levels of total IAPP in retinal and hippocampal samples (**Paper IV**) were, in addition to ELISA analysis, also analysed using dot blot analysis. Samples (0.5 μ g/ μ l) were loaded into a nitrocellulose membrane and let dry 30 min before incubating the membrane in blocking solution (BS) (5% milk powder in PBS). The membrane was then incubated with A133 as primary antibody in BS ON 4°C before rinsed and incubated with ECL goat anti-rabbit secondary antibody 1 h RT on shaker. The proteins were visualized using a BioRad CCD camera.

3.1.9 Quantification of number of pericytes and vessel length

The number of NG2+ pericytes, laminin+ pericytes and length of vessels in the molecular layer of hippocampus (**Papers I and IV**) as well as the number of NG2+ pericytes and the length of vessels in retina (**Paper IV**) were analysed by capturing images of the analysed areas using CellSens Dimension software. The number of pericyte bodies was counted and the length of vessel was measured, by a blinded observer in two pictures from three sections (six fields in total). The number of pericytes per vessel length was thereafter calculated.

The number of IAPP inclusions (**Paper III**) was scored by two blinded independent observers in five pictures of each individual. Pericytes containing IAPP (**Paper III**) were defined as cells with IAPP inclusions enclosed by NG2/laminin and analysed using confocal microscopy.

3.1.10 Quantification of NG2 immunoreactivity intensity

The NG2 immunoreactivity in pericytes with intracellular inclusions of IAPP (**Paper III**) was assessed in pictures taken in a fluorescent microscope and analysed using Zen 2009 software. Pericytes containing IAPP were chosen in the red channel (IAPP) and pictures were then taken in the green channel (pericyte marker NG2). Twenty pericytes with IAPP inclusions and 20 pericytes without IAPP inclusions were analysed and each taken picture contained one pericyte with and one without IAPP inclusions in order to compare the intensity without potential fading differences between pictures.

3.2 Cells

3.2.1 Cell cultures

The in vitro studies were conducted on fetal human brain vascular pericytes (HBVP) (ScienCell Research Laboratories) (**Papers I, II and III**), which were obtained, in compliance with local, state and federal laws and regulations, from donors who signed informant consent. The cells were cultured as monolayers in pericyte medium (PM) supplemented with fetal bovine serum (2%) (FBS), pericyte growth supplement and (1%) penicillin/streptomycin solution (1%) (ScienCell Research Laboratories) in humidified air with 5% CO₂ in 37°C. For pericyte identification, Ki67 proliferation analysis, IAPP internalization and autophagy analysis, cells were grown in poly L-lysine (PLL) coated 8-wells chamber slides (Thermo Fischer) (**Papers I, II and III**) and fixed 15 min in PFA (2%). Cells were grown as

monolayers in PLL coated 96-wells plates with clear bottom and white wall (Sigma-Aldrich) for caspase 3/7 activity analysis (**Paper III**), and in PLL coated 12-wells or 96-wells plates (Sigma-Aldrich) for cytotoxicity analysis, NG2 analysis, MMP quantification and activity assays (**Papers I, II, III**).

3.2.2 Cell identification

The morphology of our cultured HBVPs corresponded well to previously published images of HBVPs, but to further confirm we immunofluorescent stained them against the pericyte markers NG2 and PDGFR- β (**Papers I and II**) and the percentage of DAPI+/NG2+/PDGFR- β + cells of total DAPI+ cells (n=300, **Paper I**) and (n=250, **Paper II**) were approximately 99%.

3.2.3 Immunofluorescence (IF) procedures of cell cultures

Cell cultures (**Papers I, II, III**) were immunofluorescent stained by 1 hour incubation with blocking solution (BS) containing 1% bovine serum albumin (BSA) in PBS. In stainings against intracellular markers (α -SMA and Ki67) and laminin α 5, the BS was supplemented with Saponin (0.1 %) in order to permeabilize the membrane. The cells were incubated with primary antibodies (Table 1) in BS for 1 hour RT, followed by incubation with appropriate fluorescence labelled secondary antibodies in BS RT for 1 hour in the dark. Finally, cells were mounted with Vectashield Set mounting medium containing DAPI (Vector Laboratories).

3.2.4 Synthetic A β 42 preparations

Oligomer- and fibril-enriched preparations of synthetic A β 42 (**Paper II**) were prepared by dissolving the A β 42 peptide (Bachem) in cold hexafluoro-2-propanol (HFIP)(Sigma-Aldrich), aliquoted, speed vacuum dried and stored at -80°C until used. The A β peptide was then dissolved in DMSO to a concentration of 2.5 mM and sonicated 10 minutes. In order to receive oligomer-enriched preparations of A β 42, the A β /DMSO was further diluted with phenol-red free Dulbecco modified Eagle medium (Gibco) to a concentration of 100 μ M and incubated 24 hours at 4°C. To generate fibril-enriched preparations of A β 42, the A β /DMSO was diluted to 100 μ M in 10 mM HCl and incubated for 24 hours in 37°C. Prior cell experiments, oligomer- and fibril-enriched A β 42 preparations were diluted in PM to the working concentration of 10 μ M. Differences between A β 42 preparations in (**Paper II**) were documented by Western blot analysis (described below).

3.2.5 Recombinant A β 40 and A β 42 preparations

The recombinant A β 40 and A β 1-42 peptides (AlexoTech) (**Paper I**) were dissolved in HFIP and aliquoted in a similar way as described above for synthetic A β 42. The A β 40 and A β 42 peptides were then dissolved in 10 mM NaOH pH 11, and the pH was thereafter adjusted to pH 7.0 by diluting the solution in phosphate buffer to a final concentration of 100 μ M. Oligomer-enriched preparations of A β 40 and A β 42 were generated by 20 min incubation RT with agitation and fibril-enriched preparations of A β 40 and A β 42 were generated by 72 hours incubation in 37°C with agitation. To receive monomer preparations of A β 40 and A β 42, the peptides were dissolved in DMSO to a concentration of 2.5 mM and then diluted further in phosphate buffer to a final concentration of 100 μ M. Prior cell experiments, oligomer- and fibril-enriched A β 40 and A β 42 preparations were diluted in PM to the working concentration of 10 μ M. Differences between A β 40 and A β 42 preparations in (**Paper I**) were documented by oligomer analysis, fibril analysis and TEM analysis (described below).

3.2.6 Synthetic IAPP preparations

Synthetic IAPP (Bachem) (**Paper III**) was dissolved in HFIP and aliquoted as described above for A β peptides. Oligomer- and fibril-enriched preparations (EP) of IAPP were generated according to the same procedures as mentioned above for oligomer- and fibril-enriched preparations of recombinant A β 40 and A β 1-42. Oligomer-enriched preparations of fluorescein amidite (FAM)-labelled IAPP (Bachem) (**Paper III**) were generated by dissolving the peptide directly in NaOH/phosphate buffer and left to aggregate with normal synthetic IAPP (ratio 1:5) according to protocol for oligomer preparations mentioned above. Prior cell experiments, oligomer- and fibril-enriched IAPP and oligomer-enriched FAM-labelled IAPP preparations were diluted in PM to the working concentration of 10 μ M. Differences between IAPP preparations in (**Paper III**) were documented by Thioflavin T (ThT) fibril analysis and Western Blot (described below).

3.2.7 Oligomer analysis

The presence of oligomers in our A β 1-40 and A β 1-42 preparations (**Paper I**) was confirmed using an indirect ELISA with an oligomer monoclonal antibody (OMAB) as detection antibody according to previously published protocol (238). A clear 96 wells plate was coated with OMAB antibody (Table 1) ON 4°C, washed and incubated with blocking solution (5% milk in PBS) 1h RT before incubating A β 1-40 and A β 1-42 preparations (5 μ M) 1h RT. The plate was rinsed, incubated with Resco (Table 1) 1h RT and then incubated with secondary antibody (Table 2). TMB

substrate solution was used to detect HRP activity and the absorbance was measured at 450/540 nm using Biotek EON Microplate Reader. The intra- and inter-assay coefficient of variance (CV%) were <9 and <10, respectively.

3.2.8 Fibril analysis

The presence of β -sheets in our A β 1-40 and A β 1-42 preparations (**Paper I**) was confirmed by Thioflavin T (ThT) assay. The A β 1-40 and A β 1-42 preparations (5 μ l) were incubated with 40 μ M ThT solution (Millipore) in 20 nM phosphate buffer (pH 7.4, 150 nM NaCl). The signals were detected using a Microplate Spectrophotometer Infinite M200 at an emission of 435 nm and an excitation of 480 nm.

3.2.9 Transmission electron microscopy (TEM) analysis

The presence of oligomers and fibrils in our A β 1-40 and A β 1-42 preparations (**Paper I**) was, in addition to OMAB ELISA and ThT analysis, confirmed by transmission electron microscopy (TEM). The TEM analysis was performed by our collaborators at the Umeå Core Facility Electron Microscopy, the Chemical Biological Centre, Umeå University, Sweden. The A β 1-40 and A β 1-42 preparations (3.5 μ l) were absorbed onto glow-discharged carbon-coated copper grids before washed with water and stained in uranyl acetate solution (1.5 %) for 30 s. A JEM1230 transmission electron microscope (JEOL) at 80 kV was used to examine negatively stained samples and transmission electron micrographs were recorded using Gatan UltraScan 1,000 2k x 2k pixel CCD camera and Digital micrograph software.

3.2.10 Western blot analysis

The differences between oligomer-EP and fibril-EP of A β 1-42 (**Paper II**) and IAPP (**Paper III**) were verified by separating the preparations on 10 % Tris-Tricine gels and transferred to a PVDF membrane. The membrane was blocked using 5% milk powder in PBS, incubated with primary antibody in BS ON 4°C before rinsed and incubated with ECL secondary antibody 1 h RT on shaker. The proteins were visualized using a LAS-3000 CCD camera (**Paper II**) or a BioRad CCD camera (**Paper III**).

3.2.11 Cell treatment

Two hours prior cell experiments, medium was removed and replaced with serum-free medium in order to synchronize cells into the same cell cycle phase. After serum-starvation, medium was again removed and replaced with either serum-free medium (cytotoxicity experiments, (**Papers I, II and III**), NG2 experiments (**Papers II and III**), MMP experiments (**Paper II**)) or serum-supplemented medium (proliferation experiments (**Paper I**) and autophagy and IAPP internalization experiments (**Paper III**)) containing either 10 μ M fibril-EP A β 1-40 (**Paper I**), 10 μ M oligomer-EP A β 1-40 (**Paper I**), 10 μ M monomer A β 1-40 (**Paper I**), 10 μ M fibril-EP A β 1-42 (**Papers I and II**), 10 μ M oligomer-EP A β 1-42 (**Papers I and II**), 10 μ M monomer A β 1-42 (**Paper I**), 10 μ M fibril-EP IAPP (**Paper III**) or 10 μ M oligomer-EP IAPP (**Paper III**). Cells exposed for the different stimuli were compared to cells exposed for their respective controls, that is vehicles used for oligomer- and fibril-EP A β 1-40, A β 1-42 and IAPP (NaOH/PBS (**Papers I and III**)), vehicle used for oligomer-EP A β 1-42 (DMSO/DMEM (**Paper II**)), vehicle used for fibril-EP A β 1-42 (DMSO/HCl (**Paper II**)) or vehicle used for monomer A β 1-40 and A β 1-42 (DMSO/PBS (**Paper I**)). If nothing else is stated, all experiments are performed in duplicates and repeated independently three times. Cell culture supernatants were collected after treatment, centrifuged (275 x g 5 min, 4°C), aliquoted and stored at -80°C until used. Cells were lysed using cell lysis kit (Sigma-Aldrich), aliquoted and -80°C until used.

3.2.12 Cell cytotoxicity tests

3.2.12.1 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) assay kit (Sigma Aldrich) was used according to manufacturer's instructions (**Papers I, II and III**) to determine cell cytotoxicity after treatment with A β 1-40 (**Paper I**), A β 1-42 (**Papers I and II**) and IAPP (**Paper III**). This method is commonly used as necrotic cell cytotoxicity assay as LDH enzyme is released from when the cell plasma membrane is damaged/ruptured (239).

3.2.12.2 Caspase 3/7 activity

Caspase 3/7 activity assay kit (Promega) was used according to manufacturer's instructions as an additional cell cytotoxicity assay (**Paper III**) to determine whether IAPP induce apoptotic cell death. The executor caspase 3 becomes activated in programmed cell death and is therefore commonly used as a marker to distinguish programmed cell death from other types of cell death.

3.2.13 Proliferation assay using Ki67

Alterations in proliferation after treatment with A β 1-40 and A β 1-42 (**Paper I**) was determined by immunofluorescent staining of HBVPs against the cellular proliferation marker Ki67. This marker is commonly used as a proliferation marker as it is present within the cell during all active cell cycle phases (G₁, S, G₂ and mitosis) but not during the resting G₀ phase (240). Proliferation was analysed by counting the number of Ki67 positive cells of total DAPI positive cells.

3.2.14 NG2 analysis

Levels of NG2 in cell supernatants and lysates (**Paper II**) were analysed using an in-house developed assay previously described (154). Cell supernatants and lysates were coated in MesoScale Discovery high-bind plates and incubated ON at 4°C, blocked with BS (1% BSA and 1% milk PBS-T) for 1 hour. The wells were thereafter incubated with mouse anti-NG2 (kind gift Dr William Stallcup) for 2 hours at RT before incubation with mouse sulftag-conjugated secondary antibody (MesoScale) for 1 hour at RT. MesoScale Discovery SECTOR imager 6000 was used to quantify the electrochemilumiscence signal. The intra- and inter-assay coefficient of variation (CV%) in this ELISA have previously been shown to be <10 and <23 and recovery of spiked standards into CSF was between 92-107% (154).

3.2.15 MMP-9 and MMP-2 activity assay

Activities of MMP-9 and MMP-2 in cell culture supernatants from HBVPs exposed for A β 1-42 (**Paper II**) were analysed using SensoLyte Plus MMP-9 and MMP-2 assay kits (AnaSpec) according to manufacturer's instructions. The fluorescence was measured at an excitation of 490 nm and an emission of 520 nm using the Microplate Spectrophotometer Infinite M200.

3.2.16 MMP-1/3/9 analysis

Levels of MMP-1, MMP-3 and MMP-9 (both the active and the proenzyme form) in cell culture supernatants from HBVPs exposed for A β 1-42 (**Paper II**) were analysed using Human MMP 3-Plex Ultra-Sensitive kit (MesoScale). MesoScale Discovery SECTOR imager 6000 was used to quantify the electrochemilumiscence signal and detection limits for MMP-1, MMP-3 and MMP-9 were 3.15, 3.39 and 11.8 pg/ml, respectively.

3.2.17 Autophagy

Autophagosome formation after exposure to IAPP was analysed using the BacMam LC3 fluorescent protein chimera together with high transduction efficient BacMam according to manufacturer's protocol. The fluorescence acidotropic probe LysoTracker Red DND-99 (Invitrogen), sensitive for acidic organelles, was used to mark lysosomes. The cells were then fixed with PFA (2%) before examined using fluorescent microscope. Alterations in autophagosome formation were investigated by analysing pictures of HBVPs randomly selected in the DAPI channel. Increased autophagosome formation was defined as the number of HBVPs with more than 5 LC3B-FP positive dots divided by total number of cell nuclei (on average 500 cells per stimuli). Lysosomes were considered as enlarged if the diameter was larger than 3µm (more than twice the size of a regular lysosome). The percentage of co-localization (Mander's coefficient) between LC3 positive autophagosomes and lysosomes, indicative of formation of autolysosomes and functional autophagy, was analysed using confocal microscopy (Zeiss LCM 510) and calculated using Zen 2009 software.

3.2.18 IAPP internalization

Intracellular distribution of IAPP was analysed by exposing HBVPs to oligomer-EP FAM-labelled IAPP for 14h. The distribution of the fluorescently labelled IAPP in relation to lysosomes was investigated by adding the lysosomal marker lysotracker Red DND-99 the last 2h of IAPP incubation. The cellular localization of FAM-labelled IAPP and the percentage of co-localization (Mander's coefficient) between FAM-labelled IAPP and lysosomes or DAPI+ nuclei in 10 HBVPs were analysed using confocal microscopy (Zeiss LCM 510) and Zen 2009 software.

3.2.19 Microscopy

All bright field images were taken using an Olympus BX41 light microscope equipped with Olympus DP72 camera, CellSens Dimension and ImageJ software. Fluorescence images were taken using the same microscope and camera or using confocal microscope (Zeiss LCM 510) equipped with Zen 2009 software.

3.3 Statistical analysis

All statistical analyses are performed using SPSS software. Kolmogorov-Smirnov test was used to assess normal distribution (**Papers I, III and IV**) and differences and correlations were considered significant at $p < 0.05$ (**Papers I, II, III and IV**).

In **Paper I**, differences in pericyte numbers, vessel length and the ratio pericytes/vessel length were normally distributed and therefore analysed using independent samples t-test. Levels of A β 1-40, A β 1-42 and the A β 1-42/40 ratio were not normally distributed and thus analysed using Mann-Whitney U-test. Correlations between brain variables were performed using 2-tailed Spearman correlation test. Differences between oligomer-EP and fibril-EP of A β 1-40 and A β 1-42 and Ctrl O/F in the in vitro study were analysed using ANOVA, followed by Dunnett's post hoc correlation (comparisons for n=4) whereas differences between monomer A β 1-40 and A β 1-42 and Ctrl M were analysed using student t-test.

In **Paper II**, differences between oligomer-EP and fibril-EP of A β 1-42 and their respective controls were analysed using paired t-test.

In **Paper III**, IAPP cell inclusions were not normally distributed and comparisons between NCs and AD patients were analysed using Mann-Whitney test. Differences in NG2 immunoreactivity intensity were analysed using paired t-test and differences between oligomer-EP, fibril-EP IAPP and Ctrl O/F in the in vitro study were analysed using ANOVA, followed by Bonferroni post hoc correction (comparisons for n=3).

In **Paper IV**, differences were analysed using Students t-test and correlations were analysing using either 2-tailed Pearson correlation test or 2-tailed Spearman correlation test.

4. Main results

A summary of the results is presented here; the reader is referred to the original article for specific details.

4.1. Paper I

Both the A β 1-40 and the A β 1-42 peptide is associated with AD; A β 1-42 as the major component in extracellular senile plaques and A β 1-40 since it deposits in the vessel walls. Due to the differences in deposition pattern between these two A β species, **Paper I** aims to investigate whether these two A β species, in different aggregation forms, also affect brain pericytes differently and if they are involved in the pericyte loss shown in previous studies.

4.1.1. Brain tissue studies

To verify previous studies and investigate whether the hippocampal pericyte population is affected in the AD patients included in our cohort, we immunohistologically stained human post-mortem hippocampal brain tissue from AD patients and non-demented controls (NCs) against the pericyte marker NG2. The use of our pre-fixation protocol (see material and methods) allowed us to visualize nicely stained and easily distinguishable pericyte cell bodies and vessels. We counted the number of NG2+ pericytes and found significant reduced number of the same (Figure 5A) in AD patients. The significance remained even after the vessel length was taking into consideration, as we found significant fewer NG2+ pericytes per vessel length in AD patients compared to NCs (Figure 5B). We also stained the hippocampal tissue against the marker laminin, which yielded a staining where pericytes and vessels were clearly seen. However, the number of laminin+ pericytes were, in contrast to NG2+ pericytes, not altered in AD compared to NC, regardless of whether vessel length was accounted for or not (Figure 5C and D). This differences in results should be viewed from the fact that NG2 and laminin are expressed by pericytes with different properties, where NG2 are expressed by activated pericytes on capillaries/arterioles (241) and laminin on mature pericytes positioned at capillaries, arterioles and post-capillary venules (103).

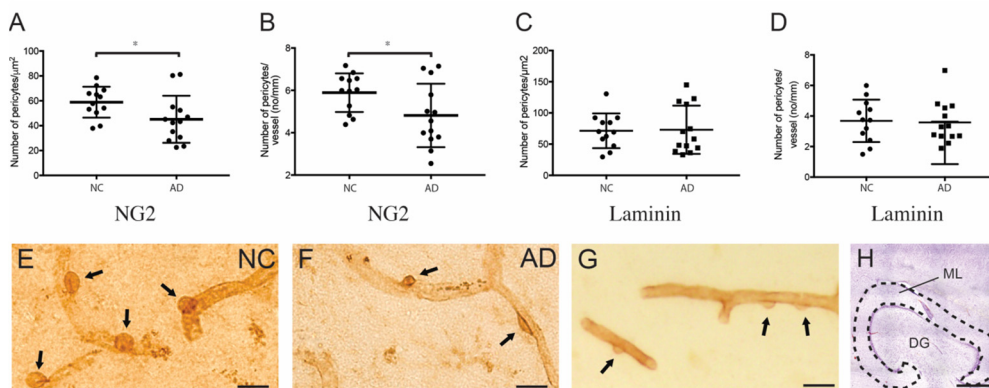


Figure 5.

Column scatter plots (A and B) demonstrate reduced number of NG2 positive pericytes/ μm^2 (A) and reduced number of NG2 positive pericytes/ vessel length (B) in hippocampus from patients with Alzheimer's disease (AD) compared to non-demented controls (NC). Scatter plots in (C and D) demonstrated unaltered number of laminin positive pericytes/ μm^2 (C) and unaltered number of laminin positive pericytes /vessel length in hippocampus from AD patients and NCs. Image in (E) shows NG2 positive hippocampal pericytes in NCs (indicated by arrows) and AD patients (indicated by arrow in F). Image in (F) is a representative picture of laminin positive hippocampal pericytes (indicated by arrows) and image in (H) demonstrate the molecular layer (ML) of hippocampus where pericytes and vessels were analysed.

In order to analyse the relationship between changes in the pericyte population and the cerebral A β load, we analysed levels of both A β 1-40 and A β 1-42 in the hippocampal tissue. We found a trend towards increased levels of A β 1-42 and higher A β 1-42/40 ratio in AD patients (data not shown). No significant alterations were found in A β 1-40 levels between the two groups. However, we found that the number of NG2+ pericytes was positively associated with levels of A β 1-40 both across the groups but especially in AD patients (Figure 6A). This association remained significant when the vessel length was taken into account (Figure 6B). No correlations between levels of A β 1-40 and any of the laminin+ pericyte variables were found, suggesting A β 1-40 to affect NG2+ pericytes in a greater extent than laminin+ pericytes. Moreover, no correlations between any pericytes variables and levels of A β 1-42 were found, suggesting that alterations in the pericyte population in AD are foremost linked to A β 1-40 and not A β 1-42.

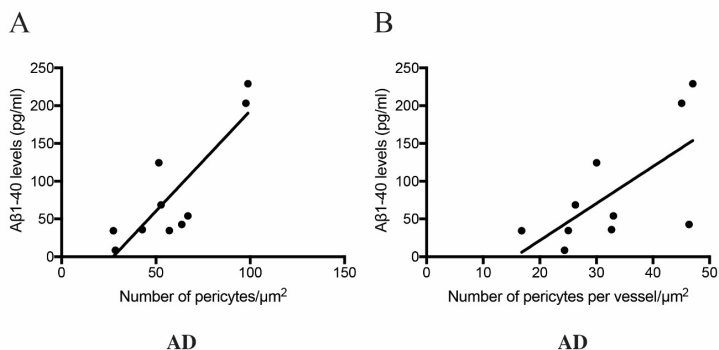


Figure 6.

Scatter plots with linear regression lines demonstrate the correlation between hippocampal Aβ1-40 levels and number of NG2 positive pericytes/μm² in AD patients (A) and the correlation between hippocampal Aβ1-40 levels and number of NG2 positive pericytes/vessel length in AD patients.

4.1.2. Cell culture studies

As our brain tissue studies indicated that levels of Aβ1-40 are linked to alterations in the pericyte population, we wanted to investigate the direct impact of Aβ1-40 on brain pericytes. We therefore exposed cultured human brain vascular pericytes (HBVPs) for different aggregation forms (monomer, oligomer and fibrils) of Aβ1-40. Interestingly, we found increased LDH activity, indicative of necrotic cell death, after stimulation with fibril Aβ1-40 compared to its control (Figure 7A), but decreased cell death after stimulation of monomer Aβ1-40 (Figure 7B) both after 24h and 96h exposure. Oligomer Aβ1-40 had no effect on pericyte viability (Figure 7A) regardless of exposure time. In order to investigate any potential species difference between Aβ1-40 and Aβ1-42, we exposed HBVPs to the same aggregation forms of Aβ1-42. No alterations in cell viability were seen after exposure to any aggregation forms of Aβ1-42 after 24h. However, increased cell death was seen after prolonged (96h) exposure to oligomer and fibril Aβ1-42 (data not shown).

In order to investigate the signal pathway underlying the Aβ induced cell death, we analysed caspase 3/7 activity after exposure to the three aggregation forms of Aβ1-40 and Aβ1-42. Our results demonstrate increased caspase activity after exposure to fibril Aβ1-40 (Figure 7C) and decreased caspase activity after monomer Aβ1-40 (Figure 7D) whereas oligomer Aβ1-40 and all three aggregation forms of Aβ1-42 resulted in unaltered caspase activity (Figure 7C and D).

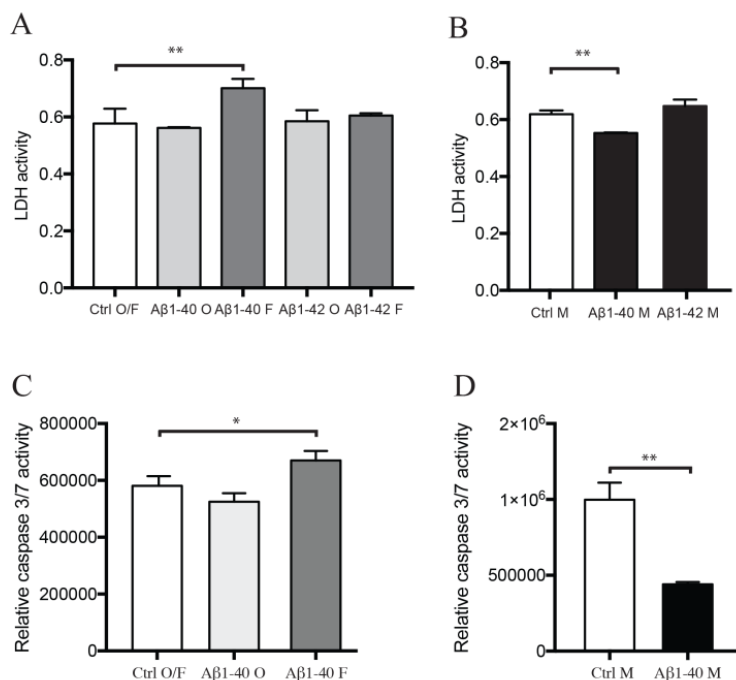


Figure 7.

Bar graphs showing alterations in LDH activity (indicative of necrotic cell death) in supernatants from HBVPs after 24 hours exposure to monomer, oligomer and fibril Aβ1-40 and Aβ1-42 (A and B) and alterations in relative caspase 3/7 activity, indicative of apoptotic cell death in HBVPs after exposure to monomer, oligomer and fibril Aβ1-40 (C and D). Graph in (A) demonstrate increased LDH activity after exposure to fibril Aβ1-40 compared to Ctrl O/F. Bar graphs in (B) demonstrate decreased LDH activity after exposure to monomer Aβ1-40 compared to Ctrl M. Bar graphs in (C) show increased relative caspase 3/7 activity in HBVPs after exposure to fibril Aβ1-40 compared to Ctrl O/F. Bar graphs in (D) show decreased relative caspase 3/7 activity in HBVPs after exposure to monomer Aβ1-40 compared to Ctrl M.

Finally, we detected increased proliferation in HBVPs exposed to monomer Aβ1-40 (Figure 8B) whereas decreased proliferation was seen in HBVPs exposed to fibril Aβ1-40 (Figure 8D). No differences were found in proliferation after oligomer Aβ1-40 exposure (Figure 8C) or after exposure to any of the aggregation forms of Aβ1-42 (data not shown).

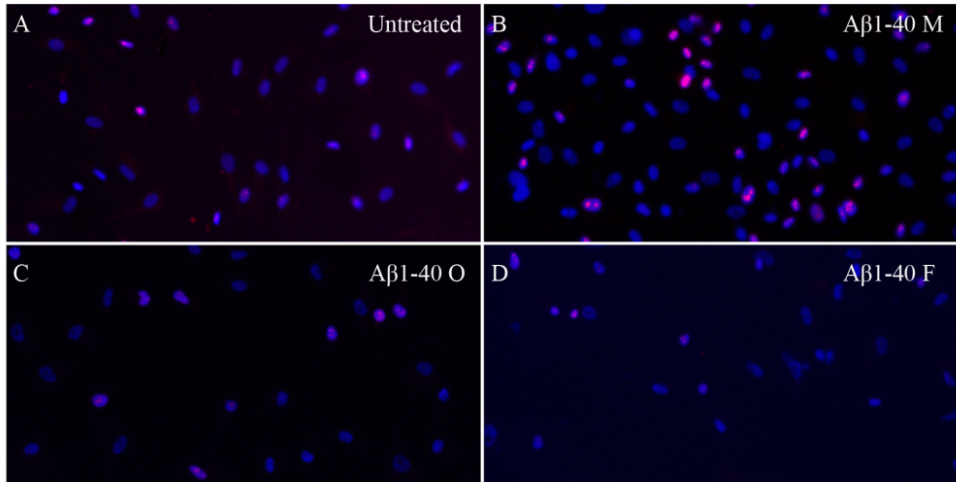


Figure 8.

Images in (A-D) demonstrate HBVPs stained against the proliferation marker Ki67. Image in (A) shows untreated HBVPs stained against Ki67. Image in (B) demonstrates monomer A β 1-40 exposed HBVPs stained against Ki67. Image in (C) demonstrates oligomer A β 1-40 exposed HBVPs stained against Ki67. Image in (D) shows Ki67-stained HBVPs after fibril A β 1-40 exposure.

4.1.3. Conclusion Paper I

In this paper, we demonstrate reduced number of hippocampal pericytes in AD patients and show that alterations in the hippocampal pericyte population are foremost associated to A β 1-40 and not the AD characteristic A β 1-42 peptide. We further use in vitro studies to demonstrate that different aggregation forms of A β 1-40 and A β 1-42 affect pericytes differently and that the fibril form of A β 1-40 is the one foremost associated with pericyte cell death.

4.2. Paper II

Even though we found no direct impact of A β 1-42 on pericyte survival and pericyte number in **Paper I**, it does not rule out the possibility that A β 1-42 affects pericytes in other ways. In **Paper II**, we wanted to investigate how A β 1-42 affects the levels of both membrane-bound and shed NG2, a proteoglycan important for pericyte survival, angiogenesis and known to be involved in long-term potentiation (LTP).

4.2.1. Cell culture studies

To investigate the impact of different aggregation forms of A β 1-42 on NG2, we exposed cultured brain pericytes to oligomer and fibril forms of the peptide and measured levels of the membrane-bound and shed NG2 (sNG2) in the cell lysate and cell culture medium, respectively. We found that levels of membrane bound NG2 were not altered, regardless of stimuli (Figure 9A). But more interestingly, levels of shed NG2 (sNG2) were significantly decreased after exposure to fibril A β 1-42 (Figure 9B). In contrast, exposure to oligomer A β 1-42 led instead to increased sNG2 compared to control. These findings indicate that different aggregation forms of A β 1-42 affect the shedding differently and may activate different pathways.

Next, we wanted to investigate the mechanisms underlying the reduced fibril A β 1-42 induced shedding of NG2. A previous study has demonstrated reduced sNG2 levels in rats after the infusion of an MMP-2/MMP-9 inhibitor, SB-3CT and suggested MMP-9 to be involved in the shedding of NG2 after spinal cord injury (242). We therefore wanted to find out whether MMP-9 could underlie also the A β 1-42 induced shedding of NG2 and analysed levels of sNG2 after the supplementation of SB-3CT. We found that the addition of SB-3CT did not have any effect on the reduced levels of sNG2 induced by fibril A β 1-42 as these levels remained low, but the increased sNG2 levels induced by oligomer A β 1-42 were ablated after supplementation with SB-3CT (Figure 9C). These results suggest that the oligomer A β 1-42 induced shedding of NG2 involves activation of MMP-2 or MMP-9.

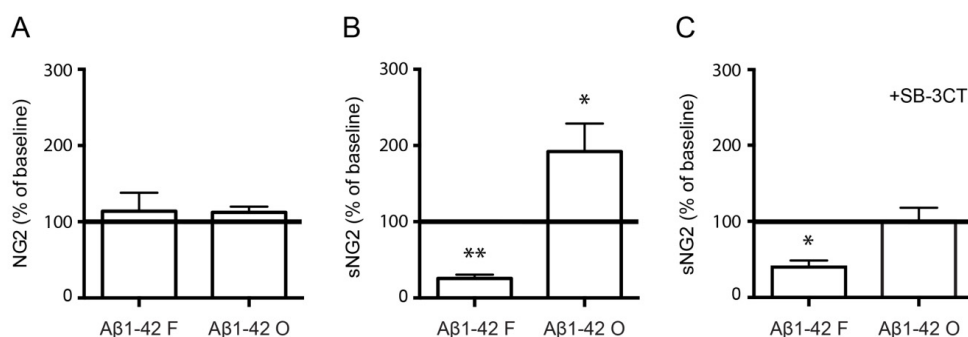


Figure 9.

Bar graphs showing alterations in NG2 in cell supernatants and lysates from HBVPs exposed for oligomer (O) and fibril (F) A β 1-42 for 24 hours in the presence or in the absence of the MMP-2/9 inhibitor SB-3CT. Bar graphs in (A) show no differences in NG2 levels in HBVP lysates after exposure to oligomer or fibril A β 1-42 compared to control. Bar graphs in (B) show decreased levels of shed NG2 (sNG2) in HBVPs supernatants after exposure to fibril A β 1-42 compared to control and increased levels of sNG2 after exposure to oligomer A β 1-42 compared to control. Bar graphs in (C) demonstrate that the increased levels of sNG2 after oligomer A β 1-42 seen in (B) were ablated by the addition of MMP-2/9 inhibitor SB-3CT. The MMP-2/9 inhibitor did not have any effect on the reduced sNG2 levels seen after exposure to fibril A β 1-42.

We therefore analysed activities of MMP-2 and MMP-9 as well as levels of MMP-1/MMP-3/MMP-9 after exposure to oligomer or fibril A β 1-42. We found reduced MMP-9 activity after fibril A β 1-42 exposure and increased, albeit not significant, MMP-9 activity after oligomer A β 1-42 (Figure 10A). We also measured levels of pro-enzyme and active MMP-9 and found reduced levels of MMP-9 after fibril A β 1-42 exposure, whereas oligomer A β 1-42 did not alter the MMP-9 levels (Figure 10B). Since the fibril A β 1-42 induced changes in MMP-9 occurred in concert with alterations in sNG2 levels, these findings support the role of MMP-9 in A β 1-42 induced shedding of NG2.

Finally, in order to conclude that the altered sNG2 levels as well as altered MMP levels and activity were not due to increased cell death in these cells, we measured LDH activity, indicative of necrotic cell death, after oligomer and fibril A β 1-42 exposure. In line with our results in **Paper I**, we found no alterations in cell death after A β 1-42 exposure, regardless of aggregation form (Figure 10C), suggesting a direct effect of A β 1-42 on the shedding of NG2 rather than an indirect effect resulting in cell death.

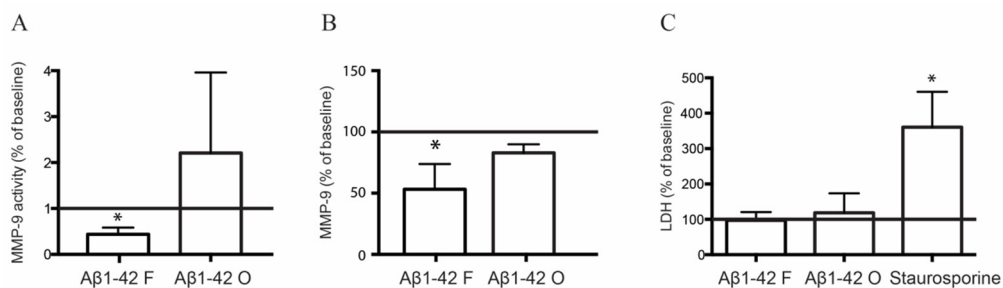


Figure 10.

Bar graphs demonstrating alterations in MMP-9 activity (A), levels of MMP-9 (B) and LDH activity, indicative of necrotic cell death (C) in HBVPs after exposure to oligomer (O) and fibril (F) A β 1-42 for 24 hours. Bar graphs in (A) show decreased MMP-9 activity after exposed to fibril A β 1-42 compared to control. Graph bars in (B) show decreased levels of MMP-9 after exposure to fibril A β 1-42 compared to control. Graph bars in (C) demonstrate unaltered LDH activity after exposure to oligomer and fibril A β 1-42 compared to control. Increased LDH activity was seen as a response after exposure to the positive control Staurosporine.

4.2.2. Conclusion Paper II

In this paper, we demonstrate that A β 1-42, in an aggregation dependent manner, affects the shedding of NG2. Our results further indicate that the A β 1-42-induced shedding of NG2 is mediated via MMP-9. The impact of A β 1-42 on sNG2 levels is particularly interesting from the perspective that sNG2 has been shown to be important for memory formation.

4.3 Paper III

Previous studies have demonstrated IAPP inclusions associated with vessels in the brain of AD patients. In **Paper III** we therefore wanted to investigate whether these IAPP inclusions are localised nearby or within pericytes and if so, how IAPP affects brain pericytes.

4.3.1 Brain tissue studies

To investigate the cellular localisation of IAPP, we stained post-mortem hippocampal tissue from AD patients and NCs against IAPP. We could clearly detect the previously described (186) IAPP inclusions (Figure 11A and B) in vessels in the molecular layer of hippocampus. By counting the number of IAPP inclusions, we could determine that there were no differences between AD patients and NCs. But interestingly, the two individuals included in the study suffering from type 2 diabetes (T2D) displayed more than twice as many IAPP cell inclusions. When we then double stained the tissue against the pericyte marker NG2 and IAPP, we could see that these IAPP inclusions were located in the cytosol of NG2 positive cells with altered cell morphology. As seen in (Figure 11C-E), cells containing IAPP inclusions displayed a blebbed/fragmented nucleus, were often closely associated to other pericytes and appeared to have a weaker NG2 staining. These findings suggest intracellular IAPP to affect the viability of pericytes as these cells displayed apoptotic features in the presence of IAPP inclusions. Finally, we also double stained the tissue against laminin and IAPP (Figure 11F), and found that these IAPP inclusions were enclosed by laminin, again suggesting these IAPP containing cells to be pericytes.

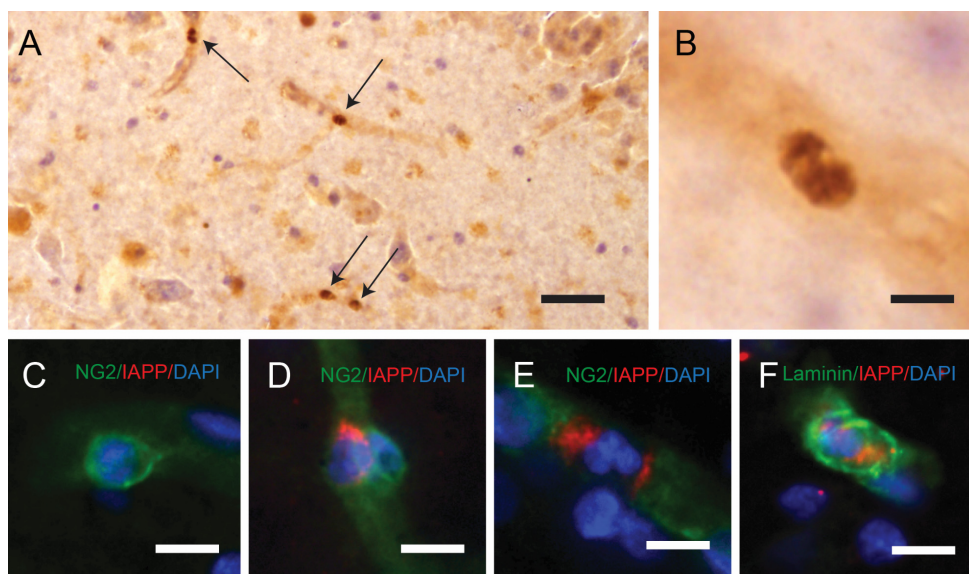


Figure 11.

Images showing Immunohistological staining against IAPP and the double staining against NG2/IAPP and laminin/IAPP in the hippocampus of an AD patient. Inclusions of IAPP are indicated with arrows in (A) and demonstrated in higher magnification in (B). A healthy pericyte with a round cell body and smooth NG2 coverage is shown in (green in C), without any IAPP inclusions (red in C), round DAPI positive cell nuclei (blue in C). Pericytes with weak NG2 staining (green in D), cytosolic IAPP inclusions (red in D) demonstrate altered cell nuclei (blue in D). Loss of NG2 coverage (green in E) was accompanied with polarized IAPP cell inclusion (red in E) and fragmented DAPI positive cell nuclei (blue in E). Cells enclosed by laminin (green in F) contained IAPP grains (red in F) and fragmented DAPI positive nuclei (blue in F).

4.3.2. Cell culture studies

In order to verify our findings in the brain tissue studies and to in more detail study the direct effect of IAPP on brain pericytes, we cultured HBVPs and exposed them to oligomer and fibril form of IAPP. We found increased LDH activity, indicative of necrotic cell death, after exposure to both aggregation forms of IAPP but with the strongest effect of oligomer IAPP (Figure 12A). In similarity to our brain tissue studies, we could detect altered/blebbed morphology of the cell nucleus linked to exposure of oligomer IAPP but also that the cell morphology was changed to a more curled up feature. To further investigate the mechanisms underlying the IAPP induced cell death in HBVPs, we analysed caspase 3/7 activity and levels of the proteoglycan NG2 in cell lysates after exposure to IAPP. We found increased caspase 3/7 activity after exposure to oligomer IAPP whereas the fibril IAPP did not affect the caspase activity (Figure 12B). These findings suggest that oligomer, but not fibril, IAPP induce cell death via a caspase dependent pathway. Furthermore, we found decreased levels of NG2 after exposure of oligomer IAPP but unaltered levels after fibril IAPP (Figure 12C). These results once again highlight oligomer

IAPP to be the more potent aggregation form of IAPP to affect pericyte function and survival.

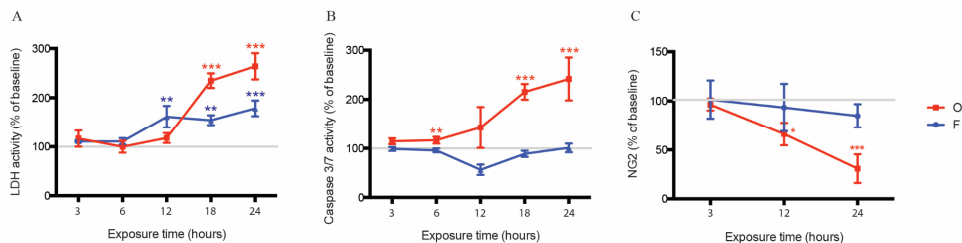


Figure 12.

Curves demonstrating alterations in LDH activity (indicative of necrotic cell death) (A), caspase 3/7 activity (indicative of apoptotic cell death) (B) and NG2 levels in lysates of HBVPs exposed to oligomer and fibril IAPP for 3,6,12,18 and 24 hours.

In order to investigate whether pericytes take up and internalize IAPP, we stimulated HBVPs with FAM-labelled IAPP. The fluorescently labelled peptide was clearly seen intracellularly in pericytes and the majority of the internalized IAPP was found outside the nuclei and co-localize with lysosomes (Figure 13A and B). The latter finding suggests that IAPP is taken up by pericytes as an attempt to degrade the toxic peptide, an idea that was tested by analysing whether IAPP exposure alter the cellular quality control mechanism autophagy. The impact of IAPP on autophagy was analysed by counting the number of LC3 positive cells since the LC3 protein is incorporated in the phagophore membrane when autophagy is initiated and the autophagosome is formed, as well as by analysing the co-localization of LC3 and the lysosomal marker LysoTracker Red DND-99. We found low number of LC3 positive autophagosomes after exposure to oligomer IAPP (Figure 14B) whereas fibril IAPP increased formation of autophagosomes already after 14 h (Figure 14C). Co-localisation analysis demonstrated low co-localisation ratio after both oligomer and fibril IAPP exposure (Figure 14E and F) but also in control-treated cells (Figure 14D). Finally, we found an increased number of enlarged lysosomes, indicative of irreversible cell injury, in cells exposed to oligomer and fibril IAPP but with the strongest effect after oligomer IAPP exposure (Figure 14E and F).

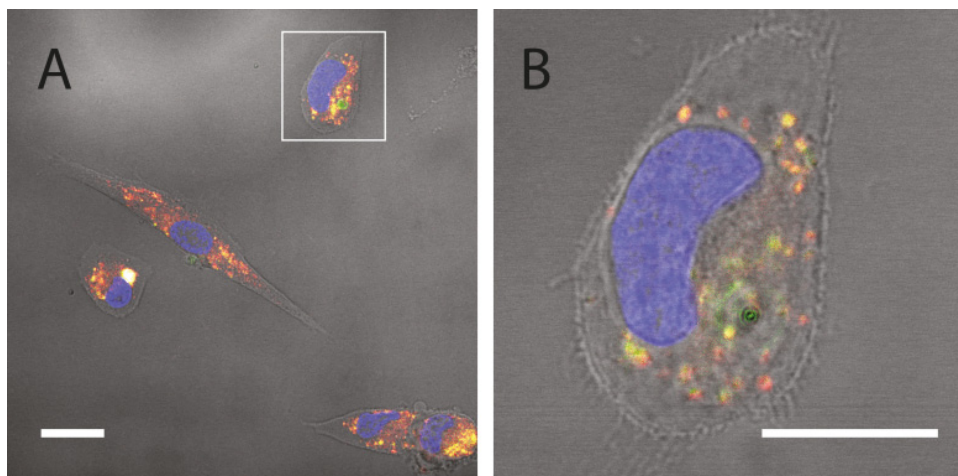


Figure 13.

Images showing IAPP internalization and co-localisation with lysosomes in HBVPs. Image in (A) demonstrates oligomer IAPP exposed HBVPs containing FAM-labelled IAPP. The co-localisation between FAM-labelled IAPP (green) and lysosomes (red) is visualized as yellow/white. Image in (B) shows a higher magnification of the HBVP in (A) indicated by the white square.

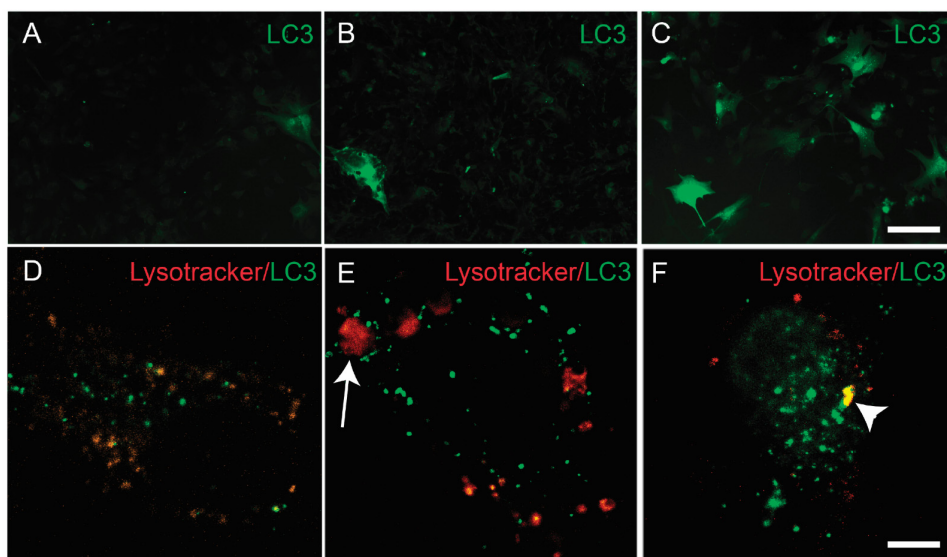


Figure 14.

Images showing autophagy in HBVPs after exposure to oligomer and fibril IAPP as well as control. Images (A and B) show the low presence of LC3 positive autophagosomes (green) after exposure to control (A), oligomer IAPP (B). Image in (C) demonstrate increased number of LC3 positive autophagosomes after exposure to fibril IAPP. Higher magnification images in (D-F) show the co-localisation of LC3 (green) and lysosomes (red) in HBVPs exposed to control (D), oligomer IAPP (E) and fibril IAPP (F). Co-localisation between LC3 and lysosomes is visualized as yellow/white.

4.3.3. Conclusion Paper III

In this paper, we demonstrate that IAPP is very toxic for brain pericytes, even more toxic than the fibril form of A β 1-40 (since the cell death is induced by IAPP already after 12 hours, whereas fibril A β 1-40 induce cell death first after 24 hours), and that the IAPP induced pericyte death is associated with dysfunctional autophagy and increased caspase activity. IAPP may also affect the pericyte response to oxidative stress via its impact on NG2, as the membrane bound form of this proteoglycan has been proposed protect cells from oxidative stress.

4.4. Paper IV

In **Paper IV**, we followed-up on our results from **Paper III** by investigating whether IAPP is present also in the retina, a highly vascularized organ considered to be an extension of the brain. We moreover wanted to study whether retinal pericytes also are affected in AD patients and finally, if the potential IAPP induced alterations in the retina can be used to mirror the IAPP induced changes in the brain.

4.4.1. Hippocampal and retinal tissue

Hippocampal and retinal tissue from AD patients and NCs were homogenized and prepared into three different fractions, S_{L-FA}, S_{U-FA}, and P_{U-FA}, in which levels of unmodified, i.e. biologically active, IAPP were analysed. Analysis of the S_{L-FA} fraction (containing most of the IAPP in the tissue regardless of aggregation form), did not show any differences in unmodified IAPP between AD patients and NCs (Figure 15A). Analysis of the second fraction i.e. S_{U-FA} (composing foremost of soluble low molecular weight IAPP) revealed increased levels of unmodified retinal IAPP levels in AD patients compared to NCs (Figure 15B). Finally, analysis of the third fraction (P_{U-FA}) demonstrated decreased levels of unmodified IAPP in AD patients compared to NCs (Figure 15C). Since the latter result coincides with previous studies demonstrating decreased levels of unmodified IAPP in blood from AD patients and given the idea that this decrease is due to a modification/aggregation of IAPP, we hypothesized that AD patients instead have high levels of modified/aggregated IAPP in the retina. Unfortunately, there are no commercially available antibodies or ELISAs detecting modified IAPP. Instead, we developed an in-house ELISA using an antibody directed toward an epitope present in both modified and unmodified IAPP. Analysis using this ELISA demonstrated unaltered levels of total retinal IAPP (P_{U-FA-G} fraction), indicating that the difference previously seen in the P_{U-FA} fraction may not lay in the levels of IAPP, but rather in the aggregation/modification form of IAPP.

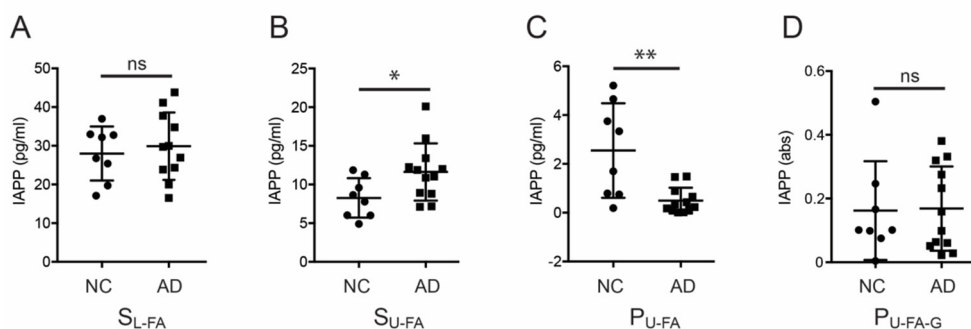


Figure 15.

Column scatterplots show changes in mean unmodified IAPP levels (A-C) and mean total, i.e. both unmodified and modified, IAPP levels (D) in different retinal fractions of AD patients and non-demented controls (NC). Column scatterplots in (A) show no differences in IAPP levels in the S_{L-FA} fraction between the two groups. Column scatterplots in (B) demonstrate increased IAPP levels in the S_{U-FA} fraction in AD patients compared to NC. Column scatterplots in (C) demonstrate reduced IAPP levels in the P_{U-FA} fraction in AD patients compared to NC. Column scatterplots in (D) show unaltered total IAPP levels in the P_{U-FA-G} fraction between the two groups.

No differences were seen in the corresponding hippocampal fractions, regardless of fraction analysed, but we detected a positive correlation between retinal and hippocampal IAPP in the S_{L-FA} fraction in NCs (Figure 16A) as well as a trend towards a correlation between retinal and hippocampal IAPP in the P_{U-FA} fraction (Figure 16B). No such correlations were found in the AD patients, but here instead a positive correlation between retinal and hippocampal IAPP in the P_{U-FA-G} fraction was found (Figure 16C). This correlation was not seen in NCs, a result suggesting that normally, i.e. in NCs, the levels of unmodified IAPP (in the S_{L-FA} and P_{U-FA} fractions) in the hippocampus are mirrored in the corresponding retinal fraction. However, in the presence of AD pathology, this relationship is disturbed, possibly due to aggregation or modifications of IAPP, and the correlation between the retina and hippocampus is instead found in the total (i.e. both unmodified and modified) IAPP levels.

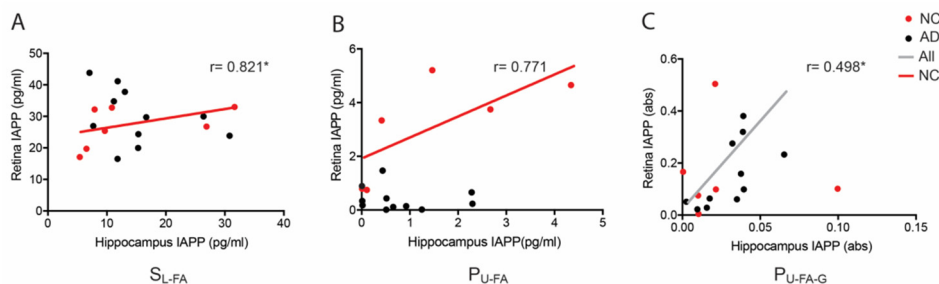


Figure 16.

Scatterplots with regression lines demonstrating the correlations between different fractions of retina and hippocampi of AD patients (black dots, black lines) and non-demented controls (NC) (red dots, red lines). Scatterplot in (A) demonstrates a positive association between retinal and hippocampal IAPP levels in the S_{L-FA} fraction in the NC group

(red dots and red line). Scatterplot in (B) shows the positive correlation between retinal and hippocampal IAPP levels in the P_{U-FA} fraction in the NC group (red dots and red lines). Scatterplot in (C) shows the positive correlation between retinal and hippocampal IAPP levels in the P_{U-FA-G} fraction across the group (grey line) and in the AD patient group (black dots).

In order to investigate the association between IAPP levels and the CNS pericyte population, we immunohistologically stained hippocampal and retinal tissue against the pericyte marker NG2 and counted the number of pericytes, measured the vessel length and calculated the number of pericytes/vessel length. We found decreased number of retinal pericytes in AD (Figure 17B), indicating that also pericytes in the retina are affected by AD pathology. No differences in retinal vessel length were seen between the patient groups (Figure 17C). The number of hippocampal pericytes was (as also shown in **Paper I**) lower, albeit not significant, in AD patients (Figure 17E). We also found a reduced length of hippocampal vessels in the same patient group (Figure 17F) (as also shown in **Paper I**), indicating that the cerebral vasculature indeed is affected by AD pathology.

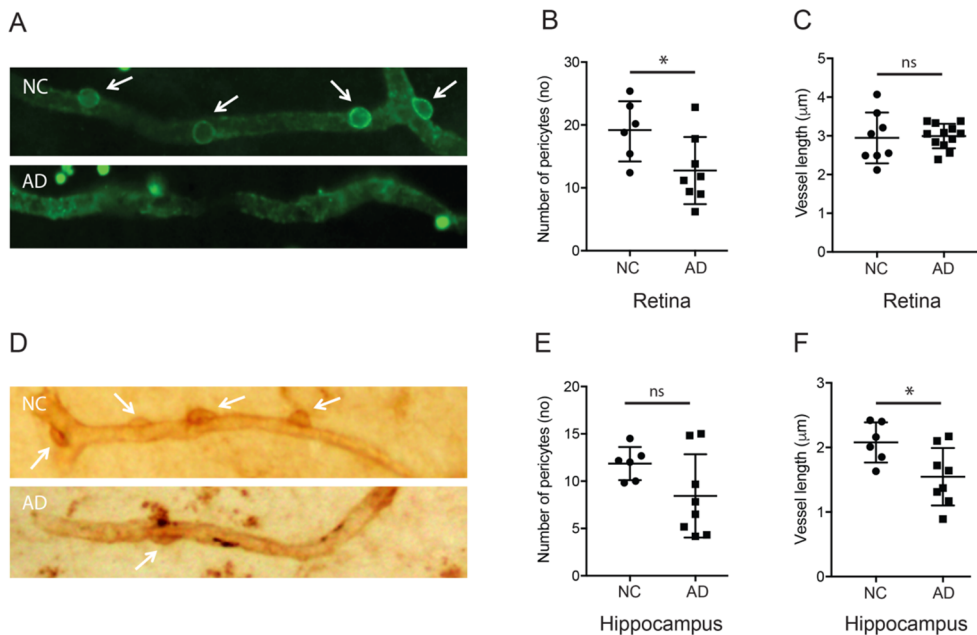


Figure 17.

Images (A and D) demonstrating NG2 positive vessels and pericytes, and column scatterplots (B, C, E, F) show the differences in mean of pericyte variables (number of pericytes and vessel length) in retinas and hippocampi of AD patients and non-demented controls (NC) when excluding Lewy body (LB) pathology. Representative images (A) of NG2 positive pericytes and vessels in retinas of NCs and AD patients. Column scatterplot in (B) shows decreased number of retinal pericytes in AD patients compared to NC. Column scatterplot in (C) shows unaltered vessel length between the two groups. Representative images (D) of NG2 positive pericytes and vessels in hippocampi of NCs and AD patients. Column scatterplot in (E) demonstrates unaltered number of hippocampal pericytes between the two groups. Column scatterplot in (F) shows reduced vessel length in AD patients compared to NCs.

Furthermore, we found a positive correlation between the number of pericytes in the retina and hippocampus in NCs but not in AD patients (Figure 18A), suggesting a functional relationship between the retinal and hippocampal pericytes in NCs, a relationship that is lost in the presence of AD pathology. Finally, we demonstrate positive correlations between the number of retinal pericytes and levels of unmodified IAPP in the S_{L-FA} fraction in AD patients (Figure 18B), suggesting unmodified IAPP to be beneficial for pericytes in AD. We also show that both in retina and hippocampus (Figure 18C and D), correlations were found across the groups between the vessel length and the IAPP levels in the S_{L-FA} fraction, indicating an impact of unmodified IAPP on the vasculature.

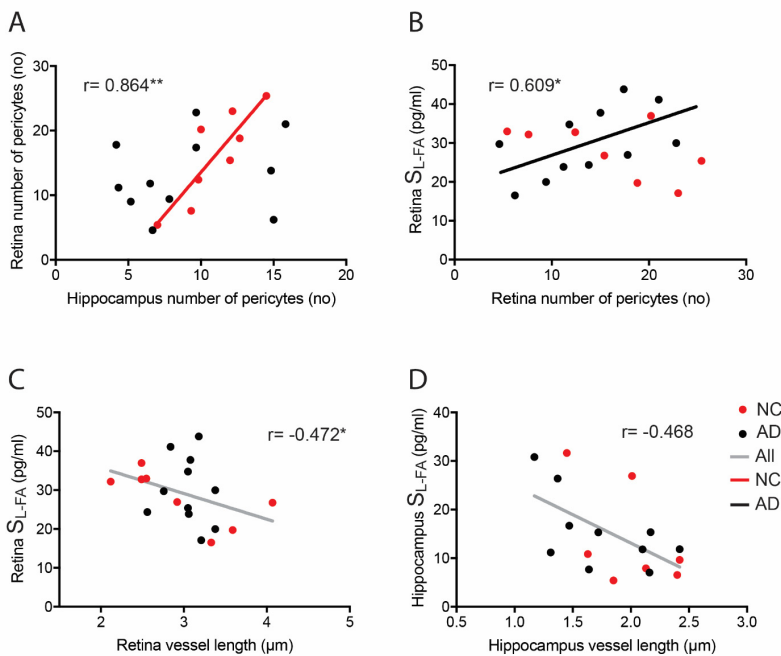


Figure 18.

Scatterplots with regression lines demonstrating the correlations between the number of NG2 positive pericytes in the retinas and hippocampi (A) as well as the IAPP levels in the S_{L-FA} fraction and the number of retinal pericytes (B) and vessel length in retinas (C) and hippocampi (D) of AD patients (black dots and black lines) and NCs (red dots and red lines). Scatterplot in (A) shows the positive correlation between number of pericytes in the retina and hippocampus of NCs (red dots and red line). Scatterplot in (B) shows the positive association between the number of retinal pericytes and retinal IAPP levels in the S_{L-FA} fraction. Scatterplot in (C) demonstrates the negative association between the retinal vessel length and the retinal IAPP levels in the S_{L-FA} fraction across the group (grey line). Scatterplot in (D) shows the negative association between the hippocampal vessel length and the hippocampal IAPP levels in the S_{L-FA} fraction across the group (grey line).

4.4.2 Conclusion Paper IV

In this paper, we show for the first time the presence of IAPP in the human retina and demonstrate a link between levels of unmodified IAPP and alterations in the pericyte population/ the vasculature. We also demonstrate that alterations in the pericyte population and IAPP levels in hippocampus correlate with the same changes in the retina, adding to the growing number of studies indicating retina as a type of window to study changes occurring in the brain.

5. Discussion

5.1 Results in context

The studies included in this thesis demonstrate that the amyloid peptides IAPP, A β 1-42 and A β 1-40 have a strong impact on brain pericytes and that this impact is aggregation- and specie-dependent. The significance of these findings and the potential mechanisms involved will be discussed below.

5.1.1 The impact of A β 1-42 on pericytes

The A β 1-42 peptide is generally considered to be the villain in AD and has therefore historically gained most attention within the AD research field. Indeed, several studies show a toxic impact of aggregated A β 1-42 on various brain cell types including neurons (243, 244) endothelial cells (245) and glial cells (246). It is therefore intriguing that our post-mortem studies (**Paper I**) and our in vitro studies (**Papers I and II**) indicate that A β 1-42 is the least pericyte-toxic variant of the three peptides analysed. It is a noteworthy finding that both fibril and oligomer A β 1-42 induced pericyte death, but only after a prolonged (96h) exposure, which is in line with previous studies demonstrating increased pericyte death after exposure to A β 1-42 for 6 days (180, 181). This rather low toxic potency of A β 1-42 regarding pericytes becomes evident when comparing with the result after IAPP and fibril A β 1-40 stimulation. Here, pericyte cell death was already observed after 12h and 24h, respectively. Despite the low toxicity, it is clear that A β 1-42 still has a strong effect on pericytes, as it in an aggregation-dependent way altered shedding of NG2 (**Paper II**). No similar impact on NG2 shedding was seen after exposure to either fibril A β 1-40 or oligomer IAPP (unpublished data). The impact of A β 1-42 on the proteoglycan NG2 is interesting with regard to pericyte function and vasculature maintenance, as it plays an important role in pericyte viability, proliferation, differentiation and angiogenesis (247, 248). The soluble version of NG2 is also interesting from an AD perspective since it has been proposed that it plays a role in the memory forming process LTP (153).

The studies in **Paper II** show that oligomer A β 1-42 enhanced pericyte shedding of NG2 via its up-regulation of MMP-9 activity, whereas the fibril form of A β 1-42 instead decreased pericyte NG2 shedding. This reduced NG2 shedding could

possibly be mediated by a reduction of MMP-9 activity. Increased activation of MMP-9 is commonly associated with neuroinflammatory processes, where activated glial cells, but also pericytes (149), secrete metalloprotease in order to degrade matrix proteins and tight junctions, which allows for migration of cells involved in the innate immune system (249). The ability of pericytes to become activated has been shown in several studies. Pericytes then start to secrete inflammatory signals (250) and differentiate into a microglial phenotype (141). In view of our findings, it appears that pericytes become activated in response to oligomer A β 1-42, since secretion and activation of MMP-9 increase. MMP-9 is also known to degrade A β 1-42 (251). Thus, it is likely that pericytes upregulate the metalloprotease as a protective mechanism against peptide toxicity. Consequently, the increase in MMP-9 also leads to downstream elevation of soluble NG2 (sNG2) levels, which enhance vascular permeability and angiogenesis as sNG2 induces migration of endothelial cells. Considering that prolonged exposure to oligomer A β 1-42 also appears to induce pericyte death, vessels consequently lose supporting cells resulting in instability and leakage. Furthermore, dying pericytes have been shown to constrict blood vessels and thereby causing permanent reduction of blood flow (252), a pathological event which could explain findings showing pericyte-associated constriction of vessels in response to oligomer A β 1-42 (184). The constriction may also impair clearance of A β 1-42 and A β 1-40 via IPAD. This impairment together with loss of pericyte-specific uptake and removal of A β 1-40 via receptors such as LRP-1 and RAGE, further promotes accumulation of the A β peptides leading to depositions characteristic of CAA (25, 158). In view of these findings, we propose the following simplified scenario of how A β 1-42 affects the vasculature (illustrated in Figure 19):

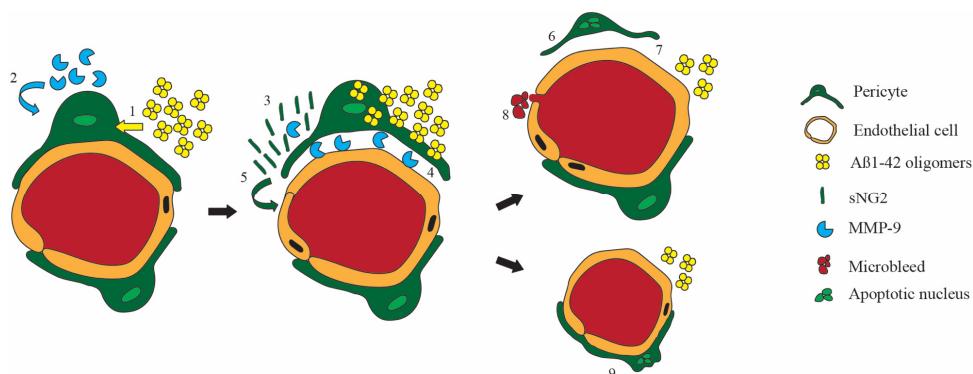


Figure 19.

Accumulation of toxic oligomer A β 1-42 leads to neuroinflammatory actions including enhanced secretion of metalloproteases (where MMP-9 is one), cytokines and chemokines from activated cells (both pericytes and glial cells). The increased MMP-9 activity leads to degradation of tight junction and increased sNG2 levels. The increased sNG2 in turn promotes migration of endothelial cells, which together with the metalloprotease-induced degradation enhance vascular permeability. After a prolonged exposure to oligomer A β 1-42 pericytes start to die, constrict the vessel and reduce blood flow as well as A β clearance. The subsequent loss of pericytes causes instability as well as the aberrant angiogenesis, dysfunctional BBB, reduced blood flow, microbleeds and vessel associated A β deposits commonly seen in AD patients.

Although our study, in line with the general opinion, suggests that aggregated oligomer A β 1-42 is harmful, it is important to emphasize that the fibril form of A β 1-42 drastically reduced NG2 shedding. This finding should be viewed from the perspective that sNG2 has been shown to be a crucial component involved in LTP. Hence, it is tempting to speculate that the fibril form of A β 1-42 may play a previously unknown role in the memory loss as observed in AD patients.

5.1.2 The impact of A β 1-40 on pericytes

The general consensus is that A β 1-40 is less harmful than A β 1-42, and some studies even suggest that this peptide is cell protective. Our results in **Paper I** show that although AD patients displayed a reduced number of pericytes, the pericyte number increased along with increased A β 1-40 levels. At a first glance this finding may suggest that A β 1-40, in line with the general idea, is beneficial for the pericyte population. It is however important to keep in mind that the impact on pericyte viability is probably dependent on the aggregating form of A β 1-40. This becomes evident when considering that vessel-associated accumulation of fibril A β 1-40, as in case of CAA, is associated the degeneration of pericytes (33). Since the tissue analysed in our study was treated with formic acid (an agent that dissolves amyloid plaques in PFA fixed tissue), we could only measure total A β 1-40 and not levels of separate aggregation forms.

The impact of the different aggregation forms of A β 1-40 was instead analysed using in vitro studies. These studies show that the three forms did indeed have different impacts on pericytes. Monomer A β 1-40 increased proliferation and reduced both caspase 3 activity and overall cell death, supporting the beneficial role of A β 1-40 previously proposed. In comparison, no alterations in proliferation were found after exposure to either monomer A β 1-42 (**Paper I**) or IAPP (unpublished data). On the contrary, fibril A β 1-40 already induced cell death after 24h, whereas A β 1-40 oligomers showed no impact on the same even after prolonged exposure. This finding is rather surprising given the well-accepted idea that amyloid peptide oligomers are the more toxic aggregation form (253). Nevertheless, the literature supports our findings suggesting that A β 1-40 might be an exception, with the fibril form of this peptide instead being the more harmful version. Several studies show that the fibril form of A β 1-40 induces degeneration of cultured neurons (177, 254, 255), endothelial cells (256, 257) and smooth muscle cells (258). Furthermore, one study has compared the toxicity of fibril and soluble (most probably both oligomers and monomers but not actually stated in the article) A β 1-40 and found cell death only after exposure to the fibrils (259). The impact of the fibril form on pericytes has not been specifically investigated, but a study showing increased pericyte death after exposure to the Dutch A β 1-40 (a version containing a mutation resulting in high fibril content (181)), indicates that the fibril-specific toxicity also applies to pericytes.

The mechanisms underlying the toxicity of fibril A β 1-40 are not fully understood. Our studies show that fibril A β 1-40-induced pericyte death is associated with increased caspase 3/7 activity. A similar finding has been presented before, where increased activation of the JNK pathway (which ultimately leads to caspase 3 activation) has been reported after fibril A β 1-40 stimulation of cultured neurons (259). Interestingly, caspase 3 activity, in studies on chemical fibrils, has also been shown to increase when engulfed and encapsulated fibrils punctuate protease-containing liposomes (260). Similar events can be expected to occur when pericytes internalize fibril A β 1-40. Another potential pathway is the binding of fibril A β 1-40 to the receptor for advanced glycation end products (RAGE) (261). Activation of this receptor is known to induce oxidative stress (262), which in turn upregulates and activates caspase 3 (263). Whether these two events are interlinked remains to be investigated, but both result in the pericyte death exemplified by degenerative pericytes associated with CAA (illustrated in Figure 20).

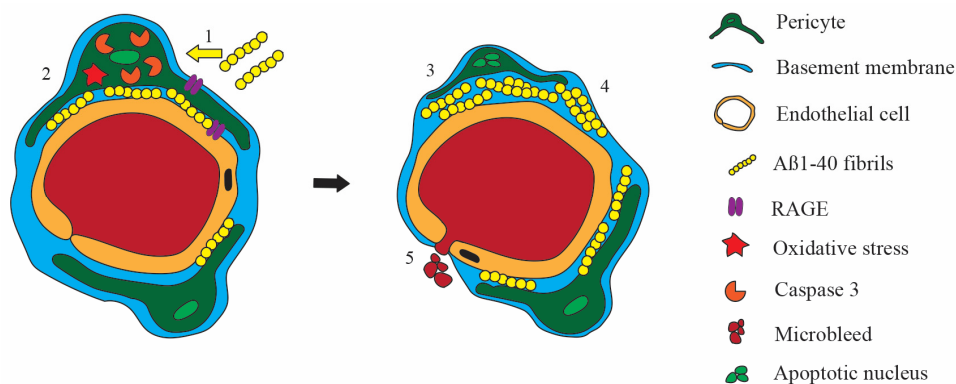


Figure 20.

1) Fibril A β 1-40 deposition associated with CAA is taken up by pericytes via RAGE, which induce 2) oxidative stress and/or lysis of caspase 3 containing vesicles. The increase caspase 3 activity induce 3) pericyte apoptosis leading to loss of clearing mechanism and 4) amplified accumulation of A β 1-40 with downstream events such as capillary stiffness and 5) microbleeds characteristic of the CAA.

5.1.3 The impact of IAPP on pericytes

The IAPP peptide is primarily associated with T2D, where the peptide is thought to underlie β -cell death linked to the disorder. However, recent studies show that IAPP depositions can be found in close vicinity to the brain vessels and it is thus tempting to speculate that the vasculature is affected by the peptide. Previous studies on the rat mesenteric arterial bed (which supplies pancreas and intestine with blood) (264) and the cat middle cerebral artery (265) have shown that IAPP influences vessel function by regulating vasodilation, however, there are no studies which investigate the impact of IAPP on vascular density and structure. Our studies in **Paper IV** show a negative association between levels of unmodified (biologically active) IAPP and vessel length in both retina and hippocampus in both AD patients and controls, indicating that unmodified IAPP has a general regulatory role in the vascular bed. A loss of such regulation could lead to pathological implications including aberrant angiogenesis and dysfunctional vessels, which are commonly seen in the AD brain, but also for example in T2D-induced retinopathy.

The negative association between levels of unmodified IAPP and vascular length could also indicate that IAPP influences vessels through impacting on cells that support the vasculature, such as pericytes. Indeed, our post-mortem hippocampal studies (**Paper III**), retinal studies (**Paper IV**) and in vitro studies (**Paper III**), show a link between IAPP and alterations in the pericyte population. The retinal studies in **Paper IV** show that the number of pericytes increases with increased levels of unmodified IAPP in AD patients, indicating a beneficial impact of biologically active IAPP on pericytes in these patients.

Although our studies highlight the interesting properties of unmodified IAPP, it is even more interesting to explore the consequences of IAPP aggregation. In **Paper III**, we demonstrate the presence of intracellular IAPP inclusions in a small fraction of brain pericytes. In addition; pericytes that contain IAPP also display nuclei with apoptotic features. These IAPP inclusions may have an extracellular origin, where peripherally-derived IAPP oligomers (266), are internalized, possibly via binding to the RAGE receptor (236). Inclusions may also arise intracellularly when internalized monomeric IAPP is allowed accumulate and aggregate (266). Which of these two events are involved, or whether both contributes to the formation of inclusions could not be determined by our observational study. However, by using an experimental in vitro approach, we showed that pericytes do internalize pre-aggregated oligomer IAPP. This led to a rapid pericyte death, a toxicity event that exceeded even the one induced by A β 1-40 fibrils. Importantly, the number of IAPP pericyte inclusions (**Paper III**) did not differ between AD and controls, but twice as many inclusions were found in the two T2D patients included in the cohort. This finding suggests that, although IAPP induces pericyte death to some extent in AD, it may not be the definitive event behind the pericyte loss seen in this disorder (25). Despite this, a loss of pericytes and thereby their A β clearance mechanisms, could accelerate A β load in an individual with an ongoing T2D pathology and thus exacerbate the progression of AD pathology. This hypothesis is supported by studies demonstrating faster cognitive decline and AD progression in T2D patients (267) (although there are studies which demonstrate the opposite i.e. slower cognitive decline in AD patients with T2D) (268). Our findings further indicate that IAPP could induce a reduction in the number of brain pericytes specifically in T2D patients, which in turn could underlie the cognitive changes associated with these patients (reviewed in (269)). Whether T2D patients display alterations in pericyte numbers in the brain has not been investigated, but the loss of pericytes in T2D in early stages of retinopathy (270), suggest that pericytes are particularly affected in this disorder.

Our studies further show that the aggregation form of IAPP determines the magnitude of caspase 3 activity and NG2 expression. In line with the generally accepted idea that amyloid peptide oligomers are usually the more toxic variant, we found a much stronger toxic impact on pericytes after oligomer IAPP stimulation compared to fibril IAPP. A similar aggregation-dependent toxicity for IAPP has been seen in studies where human islet cells display apoptotic features after exposure to freshly prepared IAPP (which quickly forms oligomers (196)), but not after exposure to fibril IAPP (271). The oligomer IAPP probably induces its toxicity via diverse pathways, but studies on β -cell death in response to oligomer IAPP specifically highlight cell membrane disruption (271-274), dysfunctional autophagy (275), oxidative stress (276) and upregulated caspase 3 activity (277). We observed dysfunctional autophagy, increased caspase 3 activity and a reduction in membrane-

bound NG2 (which has been said to increase sensitivity to oxidative stress (152), for details see **Paper III**) after oligomer IAPP exposure. Similar impact on membrane-bound NG2 was not seen after exposure to either any aggregation forms of A β 1-40 (unpublished data) or A β 1-42 (**Paper I**). It is thus likely that oligomer IAPP induces its toxicity on pericytes and in β -cells in similar ways. To summarize our findings and others, we propose a simplified scenario to explain how IAPP might contribute to cognitive impairment in T2D and exacerbate AD pathology (illustrated in Figure 21):

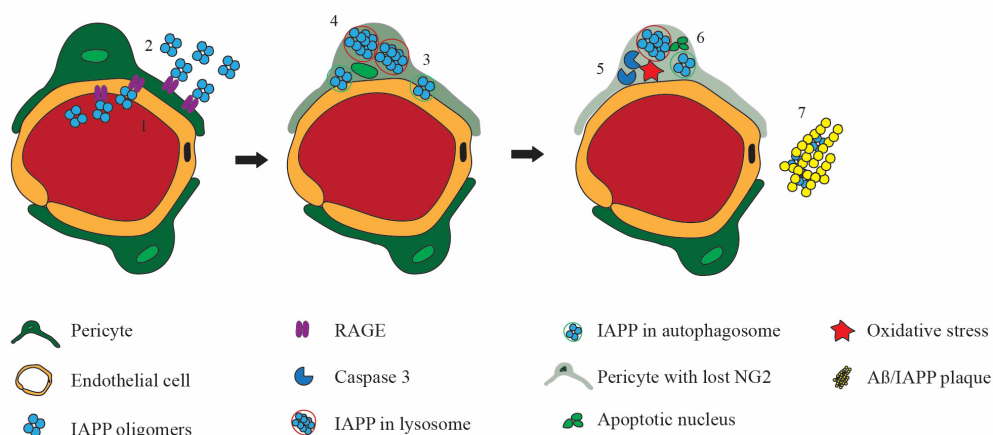


Figure 21.

Pre-aggregated IAPP oligomers from 1) the periphery or 2) from the brain parenchyma are internalized by pericytes via its binding to RAGE. The internalized IAPP is encapsulated by 3) autophagosomes (macroautophagy) and/or 4) lysosome (microautophagy) in an attempt to degrade the toxic peptide. However, the IAPP containing autophagosome fail to fuse with lysosomes, and the lysosomes which directly engulf IAPP fails to degrade the material and fuse with other similar lysosome forming enlarged lysosomes. The accumulating IAPP stress the pericytes and induce oxidative stress. 5) The reduced presence of NG2, increases the sensitivity to oxidative stress and along with a stress induced upregulation and caspase 3 activation, 6) pericytes undergo apoptosis and necrosis. The subsequent pericyte loss reduce vessel function and can 7) exacerbate accumulation of A β as the clearance mechanism of pericytes are lost.

5.2 Methodological considerations

Research studies are often accompanied by methodological limitations, which should be considered and discussed when interpreting the results achieved. Below we discuss some of the most relevant limitations involved in the methods used in **Papers I-IV**.

5.2.1 Post-mortem study limitations- cohort heterogeneity

Since AD is a disorder uniquely affecting humans (278) and great apes (279), only studies on human biological samples can provide a “true” picture of the pathologies associated with this neurodegenerative disorder. One useful approach is to perform observational human post-mortem studies, but this strategy comes with several limitations which should be considered. Firstly, individuals are unique in view of their genetic and environmental determinants. This individual uniqueness, together with the heterogeneity of neuropathology in AD cases, contribute to great variance which obstructs the distinguishing of pathological events that contribute to the disorder. Secondly, medical history and cause of death may very well lead to physiological events influencing the brain and thus add to changes which can be falsely interpreted as disorder-specific changes. Thirdly, death triggers processes, including autolysis, which in a relatively short time trigger a cascade of alterations such as transcriptional changes (280). Hence, length of post-mortem delay will influence the magnitude of changes and thereby the quality of tissue. Fourthly, post-mortem analysis only captures a snapshot at the time of death, making the approach inadequate for understanding slow progressive events as well as for distinguishing whether the observation is a cause or a result of the disorder. The first, second and third limitations can be reduced by increasing the cohort size, thereby decreasing errors and false interpretations. The cohorts analysed in the studies of this thesis were rather small, which is due to both limited access to already collected tissue from the Netherlands Brain Bank (frozen hippocampus and retina in **Papers I** and **IV**) and the fact that the protocol developed in-house (**Paper III**) requires an ongoing collection of tissue that is very time consuming. Hence, we cannot exclude the risk of over- and/or under-interpreting the results and we therefore stress that our studies need to be replicated and verified in larger cohorts. We have also, in view of the second and third limitations, excluded individuals who died as a result of traumatic events (such as skull trauma and sepsis) and exclusively chosen individuals who were autopsied within 7 h after time of death (which is generally considered as a short post-mortem delay). In view of the first and the second limitations, is it also important to consider the potential influence of cerebrovascular changes on the results, as such changes are common in the aging brain. On the other hand, as vascular disease is considered to be a risk factor for AD and individuals with vascular disease are commonly overrepresented in AD cohorts, biases can arise if such individuals are excluded. To still control for possible implications, the measured variables (pericyte no, vessel length, pericytes/vessel length) were analysed in **Paper I** after grouping the individuals based on the presence or absence of severe vascular disease. No differences between the groups were found and thus it can be concluded that the impact of A β 1-40 on the pericyte variables (i.e. pericyte numbers and vessel length) in AD patients exceeds the potential influence of other vascular complications. Other factors that could potentially have an impact on the

results and should be considered are gender, age, cholesterol and ApoE status. None of these factors were found to have an effect on the results described in **Papers I, III and IV**. The fourth limitation, i.e. the snapshot problematic, is more difficult to circumvent and we have therefore chosen to verify our observational findings in experimental in vitro studies.

5.2.2 Post-mortem study limitations- immunohistochemistry

Immunohistochemistry is often accompanied by limitations as the method is dependent on i) antibodies specific to the antigen and ii) antigen remaining intact after post-autopsy treatments and/or antigen retrieval steps. These limitations have been particularly noticeable within the research field of brain pericytes, as good commercially available antibodies directed against human pericyte markers are scarce and the few available depend on how the tissue is fixated. In the studies included in this thesis, we chose to analyse pericytes by staining against the proteoglycan NG2, principally because the proteoglycan is of interest in view of its role in memory formation, angiogenesis and oxidative stress but also as it is a well-documented marker for pericytes and an antibody with high specificity against human NG2 proteoglycan is commercially available. Importantly, NG2 is very sensitive to PFA over-fixing as well as antigen retrieval, but by fixating frozen or fresh tissue in PFA for only a short period, we managed to stain pericytes and get clearly visible cell bodies (**Papers I, III and IV**). Although, analysis of the staining revealed changes in number of pericytes (**Papers I and IV**) and cellular localization of IAPP (**Paper III**), it is important to remember that analysing only one marker restricts the methods of analysis as pericytes are known to express a variety of markers depending on their developmental stage, milieu, origin and localisation along the vasculature. Additional stainings against other pericyte markers are crucial in order to verify the results. In **Paper IV**, we also stained and analysed the pericyte population using laminin, which also yielded distinct clear pericyte cell bodies. Analysis of this staining did not show the same alterations as found after NG2 analysis, however this marker captures a slightly different pericyte population, which might explain the contradictory results. Pericytes expressing NG2 are believed to share the same properties, location and differentiation status as pericytes expressing PDGFR- β , another well-described pericyte marker. Staining against this marker would thus be of interest as a confirmatory marker. However, despite several attempts using various different antibodies and tissue treatment protocols, we were unable to get achieve reliable staining where pericyte cell bodies could be detected. This could be due to the handling and fixing of our brain tissue as others have succeeded in staining pericytes using this marker in paraffin embedded human brain tissue (25).

Another drawback associated with immunohistochemistry can be found in our retina study. Since our brain tissue study (**Paper III**) showed that pericytes occasionally contain IAPP inclusions and as we, together with others, have found an association between IAPP and vasculature/pericytes (204), we tried to stain the retina against IAPP to investigate whether similar inclusions could be found in this compartment. Unfortunately, our retinal stainings were accompanied by high background and contamination of melanin pigment, which contributed to unreliable interpretations. Despite trying various methods (including different antigen retrieval methods as well as bleaching) to reduce both background and melanin contamination, we were unable to achieve reliable stainings. Further studies and optimization of the methods are needed to establish whether IAPP also deposits or forms inclusions in the retina. Similarly, since A β depositions have been found in the retina of AD mice models and AD patients and given previous results suggesting a co-deposition of IAPP and A β in the brain, it is rational to analyse the presence of retinal A β depositions in our cohorts. Again, despite several attempts we were unable to obtain reliable A β staining due to the same problem as stated above. To still get a picture of whether A β was present or accumulated in the retina of our AD patients, we measured levels of A β 1-42 and A β 1-40 with a commonly used ELISA (MesoScale). The levels of A β 1-42 were very low (the majority of values were below detection levels) and although most samples contained detectable A β 1-40 levels, we found no significant differences between AD patients and controls (unpublished data). We can therefore in our studies not draw any conclusions about whether interactions between A β and IAPP occur in the retina.

5.2.3 In vitro model limitations - applicability

In vitro methods, using either immortalized cell lines or primary cell cultures, are commonly used to study the direct impact of a substance or event on one or more cell types. Such an approach was used to verify and further explore the findings observed in our post-mortem studies, where primary human foetal pericytes were used as a cell culture model. The model comes, however, with several limitations as single cells in a plastic dish can never replicate what happens in complex biological systems (the body). The use of cell culture models should be seen for what it is, a simplified and readily available model to direct study of isolated events. The deficiencies and drawbacks of cell culture models must be considered when performing experiments and evaluating results. In our studies, we used monolayers solely of pericytes, and it is important to consider the fact that other results might have been obtained if a co-culture of pericytes together with endothelial cells and astrocytes had been used. This is particularly true considering the potential risk of pericytes losing some of their functions if the environment is not adequate. However, pericytes and for example astrocytes can secrete the same molecules and

since we wanted to examine pericyte specific alterations in signal pathways, we chose a monolayered model design to suit our purposes better. Another limitation, which principally applies to primary cells, is the fact that the phenotype of cells changes along with increasing passage number and high cell turnover. To limit this obstacle, we only used cells in passage three or below, according to recommendations from the distributors. In vitro studies are also unable to mimic chronic events associated with human disorders and it is therefore difficult to for example to replicate accumulation of amyloid peptides over a prolonged time. To still be able to understand how accumulated amyloid peptides influence cells, high concentrations of amyloid peptides have commonly been used in in vitro studies. Notably, high concentrations are rarely physiologically relevant. The accuracy when using fetal pericytes can also be questioned, as fetal cells in theory could differ from adult cells. Hence, we cannot exclude the possibility that experiments performed on cells isolated from adult brain tissue would yield a different result. Studies comparing adult and fetal brain pericytes are scarce, but it has been shown that fetal pericytes isolated from the liver express a set-up of markers similar to adult pericytes, and there is no current evidence to contradict the assumption that similarity between fetal and adult pericytes can be expected throughout the body (281).

5.2.4 In vitro model limitations – peptide preparations

Amyloid peptides for experimental studies are commercially available in either synthetic or recombinant forms. The synthetic peptides are most widely used within the AD research field. This chemically produced peptide, however, suffers from poor reproducibility, as the production methods lead to impurities in the form of pre-aggregates which vary between batches. These pre-aggregates act as starting material determining the structural assemblies of the peptides, resulting in different compositions each time preparations are made (282). Recombinant peptides have, in contrast, proved to have better reproducibility as they do not form pre-aggregates. They are therefore believed to act in a more biologically relevant way (283). It is important to consider the differences between synthetic and recombinant amyloid peptides when comparing the effect of the three peptides on pericytes in our studies. We used recombinant peptides in **Paper I** (A β 1-42 and A β 1-40) and synthetic peptides in **Paper II** (A β 1-42) and **Paper III** (IAPP). This inconsistency in choice of peptide, together with the fact that the impact of different peptides was not examined in the same experiment, may influence our results and thereby lead to misleading interpretations. Our results in **Paper I**, where we show no impact of recombinant A β 1-42 on cell survival, did however coincide with the result found in **Paper II**, where unaltered cell viability was seen in response to synthetic A β 1-42 (regardless of aggregation form). This finding suggests that the pericytic response

to synthetic and recombinant peptides does not differ, at least not in terms of A β 1-42 toxicity.

The comparisons between peptides become even more complex as the preparations in the three papers were made according to two different protocols. The protocol used in **Paper II** was first described by Dahlgren et al. and was for a long time the most widely used protocol (177). The protocol, used in **Papers I** and **III**, has been used more frequently in recent years since it is considered to yield aggregates that are more physiologically and biologically relevant (284). Both protocols were useful for making large amounts of oligomers in our oligomer preparations and fibrils in our fibril preparations, which we showed by the use of TEM, Western blot, oligomer specific ELISA and ThT assay. These methods do not however reveal whether the aggregates formed by the two different protocols have the same properties. In order to compare the protocols, we examined whether A β 1-40 and A β 1-42 preparations yielded by the two protocols induced similar toxic responses in pericytes. We found no protocol-specific differences in results, regardless of species (A β 1-40 or A β 1-42) or aggregation forms (oligomers or fibrils) (unpublished data).

Even though the two protocols yielded the desired compositions (i.e. monomer, oligomer and fibril-enriched preparation), it cannot be ignored that the preparations were not homogenous and to some extent contained a mixture of other aggregation forms ranging from monomers to larger aggregates. In addition, amyloid peptide aggregation is favoured by higher temperatures, which means that the peptide continues to aggregate during a 24 h experiment at 37 °C incubation. The unavoidable mixture of different aggregation forms in our preparations inevitably influenced the result, where the observed impact is the sum of the effect of all aggregation forms rather than the effect of one distinct aggregation form. For example, it is quite likely that the increased pericyte death seen after prolonged exposure to fibril A β 1-42 is a result of the presence of toxic A β 1-42 oligomers in the fibril preparation. This also means that preparations containing only one aggregation form would probably yield a more distinct result with greater differences in pericyte variables.

5.3 Implications and future directives

According to the literature, pericyte loss appears to play a significant role in AD. Our studies suggest that A β 1-40, A β 1-42 and IAPP, contribute in different ways at least in part to this pericyte loss. How this new knowledge can be applied in future research into new diagnostic tools and drug targets will be discussed below.

5.3.1 Diagnostic tools

Based on the fact that AD patients form a very heterogenic patient group, it has been become evident that new knowledge, enabling different pathological causes to be distinguished, is crucial for reaching the next level of clinical diagnosis. Our studies demonstrating how three different amyloid peptides might contribute to pericyte loss and vascular alterations in AD and T2D, could be seen as one step in this direction. For example, the link between IAPP and vascular /pericyte alterations could highlight IAPP as a possible CSF biomarker for distinguishing not only IAPP-induced vascular changes in the brain of T2D patients, but also for distinguishing patients with type 2 diabetes dementia (T2DD). These patients present a cognitive decline sometimes difficult to distinguish from AD symptoms, but rarely show alterations in the classical core AD biomarkers. In addition, since it has been suggested that AD patients with T2D have a more rapid decline in cognition, it may be useful to evaluate whether this decline is linked to IAPP pathology. The application of A β 1-40 and A β 1-42 as CSF AD biomarkers is already ongoing, but their potential for distinguishing pericyte alterations is less explored. There is however one study that demonstrates reduced sNG2 along with decreased A β 1-42 (indicative of increased plaque load) in AD patients (154), which highlights sNG2 as a potential biomarker for monitoring the impact of fibril A β 1-42 on NG2 shedding and alterations in pericytes.

The fact that we also detected IAPP in the retina ought not to be controversial since IAPP is transported by blood and should therefore reach tissues with high vascularity, including the retina. However, since the levels of IAPP were negatively associated with vessel length, it can be assumed that IAPP also deposits in the retina. Such deposition could have a tremendous impact on the retinal vascular bed, which our findings in **Paper IV** also suggest. Furthermore, given that IAPP influences pericytes and the vasculature both in retina and brain, it is likely that oligomeric IAPP also affects pericytes, or cell siblings such as smooth muscle cells, in other organs. This hypothesis becomes particularly interesting in view of the fact that most complications associated with T2D involve vascular pathologies. For example, the early loss of pericytes found in diabetic retinopathy (285) might, in view of our findings, be associated with increased depositions of IAPP. If this holds true, then monitoring IAPP in the retina, possibly by means of an IAPP oligomer-specific ligand, could be a valuable tool for monitoring IAPP-induced pathology in the retina. Moreover, since our findings demonstrate that the alterations in IAPP levels and pericyte numbers in the hippocampus correlate with the same parameters in the retina, it is tempting to speculate that the retina also could be used as a type of window through which to study IAPP changes occurring in the brain (58, 59, 61, 286, 287).

5.3.2 New drug targets

Given the important role for pericytes in vascular function and maintenance, it of interest to find therapies to limit the impact of amyloid peptide on the pericyte population. Here, our studies which demonstrate the aggregation- and specie-dependent differences among the three amyloid peptides, could contribute with important knowledge. One example where differences in aggregation forms of A β 1-42 have been considered is in the development of the antibody BAN2401 (Bioartec/Biogen), which has recently been evaluated in a phase IIb clinical trial (288). While several clinical trials of immunotherapies targeting fibril A β 1-42 have failed to halt cognitive decline in AD patients, BAN2401, which targets the toxic oligomer/protofibrils of A β 1-42, has succeeded to show significant cognitive improvement after 18 months in these patients. Our studies moreover highlight that IAPP is particularly toxic for pericytes and thus this peptide could be of interest when designing new therapies against vascular complications in disorders where IAPP is dysregulated.

6. Conclusions

The studies included in this thesis use various translational and experimental approaches to analyse how the amyloid peptides A β 1-40, A β 1-42 and IAPP influence pericytes, a vessel-supporting cell type vital for blood-brain barrier integrity and clearance of brain toxic by-products. The results obtained from the studies demonstrate considerable differences in effects between different amyloid peptides on the pericyte population. This information is novel and important to consider in future research on diagnostic tools and treatments for Alzheimer's disease and disorders with vascular complications such as type 2 diabetes.

The specific conclusions drawn are:

- Patients with Alzheimer's disease display reduced number of pericytes in both retina and hippocampus.
- The size of the pericyte population in Alzheimer's disease patients are foremost associated with A β 1-40 and less with A β 1-42 or IAPP.
- All three amyloid peptides influence pericyte survival, but the toxicity varied between the peptides with the following order of decreasing toxicity: IAPP > A β 1-40 > A β 1-42.
- The three peptides act on pericytes via distinct pathways and induce different specie-specific events, which include, but are not limited to, alterations in proliferation as well as expression and shedding of NG2.
- The impact of the three amyloid peptides on pericytes proliferation, survival, NG2 expression/shedding and autophagy is dependent on the aggregation-form of the peptides.

7. Acknowledgements

A PhD is nothing you do without great support from others. There are several people that I wish to thank for standing by my side, for making this possible and for filling my PhD years with laughs and love.

First of all, I wish to express my deepest appreciation to my main supervisor **Malin Wennström**, whom without my life would not be the same. From the day I nervously entered the lab to start my bachelor work in 2012, you have guided and supported me, both professionally and personally. You have always encouraged and allowed me to explore my own ideas, no matter how crazy they have been. I cannot with words describe how thankful I am for everything you have taught me, the hours you have spent helping me, your always so wise, honest and kind words and for all the unforgettable moments and laughs we have shared around the world the past 7 years. I could not wish for a better supervisor, role model and friend!

I would also like to thank my co-supervisors; **Malin Fex**, for always being there on the other side of the street, ready to help and solve all my problems. Your broad knowledge in science and your expertise within the diabetes field has been invaluable to me. **Katarina Nägga**, for all of our interesting discussions how to interpret my results from a clinical point of view. Without you and your expertise within dementia care and research, my studies and this dissertation would not be half as good. I feel honoured and grateful to have you two inspiring women as my co-supervisors and colleagues!

My closest and finest colleagues at Wallenberg lab; **Elin Byman** for being the best colleague, room-buddie and friend one could wish for. I am truly grateful that Malin decided to adopt you from floor 4! **Camilla Orbjörn** for being as an extra mother to me. I cannot describe how thankful I am for the technical help and support you have given me during the years. And above all, the hilarious moments we have shared and all the pranks you have taught me! **Bodil Roth** for always supporting me when I needed it the most, your wise words and for always knowing the answer regardless of the question asked. **Lena Stenberg** for our many chats and discussions and for helping me out and letting me use your microscope and cryo-stat. **Marcus Ljungkvist** for always giving me a great challenge, regardless of whether it is bowling or betting games in the European/World championship.

The Netherlands Brain Bank (NBB) and especially **Michiel Kooreman**, for supplying the human brain and retina tissue and for the advice regarding pre-treatment of tissue. My studies would not be possible without you and your help, thank you!

My co-authors; **Anders Olofsson** and **Kristofer Brännström** for all the help with the amyloid peptides and your generosity regarding sharing peptides, antibodies and protocols. My amyloid preparations and my knowledge about amyloid kinetics would not be as good without you. I will always be thankful and remember the AD/PD meeting in Nice when we first met! **Simon Moussaud** for helping me with the APOE genotyping. **Henrietta Nielsen** for all the research input on my projects, for sharing your knowledge and for being an inspiration and a great role model of an independent female researcher.

The **Umeå Core Facility Electron Microscopy** at the Chemical Biological Centre, Umeå University for the technical assistance with my TEM analysis.

Gunilla Westermark for all the help and discussions regarding IAPP and for generously sharing both protocols and antibodies. Your broad knowledge and expertise, especially within the IAPP field, have been invaluable for my projects.

Arne Brun for being a great mentor and inspiration to me. Whenever I have had a question regarding neuropathological findings in the brain, you have had the answer. The things about the brain that you have not seen and don't know about, they probably do not exist. I cannot express how grateful and honoured I am to have worked with you!

Anders Tingström for the inspiration and for being the best research-grandfather.

All researcher colleagues at **Clinical Research Memory Unit** and especially **Oskar Hansson**, **Håkan Toresson** and **Lennart Minthon**. Also specific thanks to **Victoria Larsson**, **Erik Stomrud**, **Anna Svenningsson**, **Erik Nilsson**, **Emelie Andersson** and **Anna-Märta Gustavsson** for the inspiring discussions during our journal clubs.

Everyone at **Wallenberg lab floor 1** for the enjoyable lunch breaks and coffee chats we have shared during the years and for the help when struggling with cross-words! All of you at **Wallenberg lab floor 4, 5 and 6** for inspiring Thursday-meetings, Christmas parties and to **the Cake society** for keeping my blood sugar levels high by bringing delicious cakes. Special thanks to **Anna Blom** for generously letting me use your equipment and to **Tomi** and **Sara** for always helping me with equipment, protocols and antibodies.

To all former colleagues and students that I have had the privilege to work with during these years.

The PhD student's learning process was supported by the Swedish National Graduate School for Competitive Science on Ageing and Health (SWEAH) funded by the Swedish Research Council. Special thanks to **Stina Elfverson** for the continuous administrative support throughout the years.

All of my fantastic friends around the world for bringing sunshine and happiness to my life. Special thanks to **Sophie, Ida, Stina, Anneli, Sofia, Linnéa, Vendela, Jonas** and **Erik**, you are the best!

Casper and the rest of the **Kerrén** family for standing by my side the last couple of years.

My wonderful best friends and the loves of my life; **Charlotte, Alice** and **Karin** for always being there, no matter what. Without the three of you, I would be lost. Thank you for the unconditional love, friendship, laughs and support you give, I love you! **Anton** and **Filip** for the standing invitation to your homes, for the endless number of dinners and glasses of wine. My youngest best friend **Leonora**, for providing happiness, hugs and love every day.

My amazing family of uncles, aunties and cousins. Special thanks to **Cilla** and **PE** for always being there for me, and to **Staffan** and **Erika** for taking me along with you on your fantastic journeys around the world!

Lastly, but certainly not least, the most loving and generous people I know, **my family**. I am lucky to have you as my little brother, **Wilhelm**. You amaze and inspire me every day with your drive and creativity but above all, for your big heart. My amazing sister and best friend, **Nicole**, for always having my back no matter what, when or where. I hope that I one day can be as wise, loving and strong as you are. **Oskar** for being the best brother-in-law. **Mamma** and **Pappa**, for making me believe that I can achieve everything I want, every day. The two of you are my biggest inspiration in life and my safe harbour. Without your support and endless love, I would not be the person I am today. This is all for you, I love you ♥!

8. References

1. Alzheimer A. Über eine eigenartige Erkrankung der Hirnrinde. Allgemeine Zeitschrift für Psychiatrie und Psychisch-gerichtliche Medizin. 1907.
2. BrightFocus Foundation. Alzheimer's Disease: Facts & Figures [Available from: <https://www.brightfocus.org/alzheimers/article/alzheimers-disease-facts-figures>].
3. Albert MS. The ageing brain: normal and abnormal memory. Philos Trans R Soc Lond B Biol Sci. 1997;352(1362):1703-9.
4. Assal F, Cummings JL. Neuropsychiatric symptoms in the dementias. Curr Opin Neurol. 2002;15(4):445-50.
5. Hof PR, Glanvakopoulos P, Bouras C. The neuropathological changes associated with normal brain aging. Histol Histopathol. 1996;11(4):1075-88.
6. Perl DP. Neuropathology of Alzheimer's disease. Mt Sinai J Med. 2010;77(1):32-42.
7. Mormino EC, Kluth JT, Madison CM, Rabinovici GD, Baker SL, Miller BL, et al. Episodic memory loss is related to hippocampal-mediated beta-amyloid deposition in elderly subjects. Brain. 2009;132(Pt 5):1310-23.
8. Jack CR, Jr., Knopman DS, Jagust WJ, Petersen RC, Weiner MW, Aisen PS, et al. Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. Lancet Neurol. 2013;12(2):207-16.
9. Whitwell JL. Progression of atrophy in Alzheimer's disease and related disorders. Neurotox Res. 2010;18(3-4):339-46.
10. Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. Acta Neuropathol. 1991;82(4):239-59.
11. Dickson DW. The pathogenesis of senile plaques. Journal of neuropathology and experimental neurology. 1997;56(4):321-39.
12. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A. 1985;82(12):4245-9.
13. Thal DR, Rub U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology. 2002;58(12):1791-800.
14. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging. 1995;16(3):271-8; discussion 8-84.
15. Mattsson N, Smith R, Strandberg O, Palmqvist S, Scholl M, Insel PS, et al. Comparing (18)F-AV-1451 with CSF t-tau and p-tau for diagnosis of Alzheimer disease. Neurology. 2018;90(5):e388-e95.

16. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science*. 1992;256(5054):184-5.
17. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, et al. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*. 2002;416(6880):535-9.
18. Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ. Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. *J Physiol*. 2006;572(Pt 2):477-92.
19. Rabinovici GD, Jagust WJ. Amyloid imaging in aging and dementia: testing the amyloid hypothesis in vivo. *Behav Neurol*. 2009;21(1):117-28.
20. Pihlaja R, Koistinaho J, Malm T, Sikkila H, Vainio S, Koistinaho M. Transplanted astrocytes internalize deposited beta-amyloid peptides in a transgenic mouse model of Alzheimer's disease. *Glia*. 2008;56(2):154-63.
21. Wegiel J, Wang KC, Tarnawski M, Lach B. Microglia cells are the driving force in fibrillar plaque formation, whereas astrocytes are a leading factor in plaque degradation. *Acta neuropathologica*. 2000;100(4):356-64.
22. McCaulley ME, Grush KA. Alzheimer's Disease: Exploring the Role of Inflammation and Implications for Treatment. *Int J Alzheimers Dis*. 2015;2015:515248.
23. Kook SY, Seok Hong H, Moon M, Mook-Jung I. Disruption of blood-brain barrier in Alzheimer disease pathogenesis. *Tissue Barriers*. 2013;1(2):e23993.
24. Ryu JK, McLarnon JG. A leaky blood-brain barrier, fibrinogen infiltration and microglial reactivity in inflamed Alzheimer's disease brain. *J Cell Mol Med*. 2009;13(9A):2911-25.
25. Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, Zlokovic BV. Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathol*. 2013;23(3):303-10.
26. Blennow K, Wallin A, Fredman P, Karlsson I, Gottfries CG, Svennerholm L. Blood-brain barrier disturbance in patients with Alzheimer's disease is related to vascular factors. *Acta Neurol Scand*. 1990;81(4):323-6.
27. Zipser BD, Johanson CE, Gonzalez L, Berzin TM, Tavares R, Hulette CM, et al. Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging*. 2007;28(7):977-86.
28. Halliday MR, Rege SV, Ma Q, Zhao Z, Miller CA, Winkler EA, et al. Accelerated pericyte degeneration and blood-brain barrier breakdown in apolipoprotein E4 carriers with Alzheimer's disease. *J Cereb Blood Flow Metab*. 2016;36(1):216-27.
29. Janelidze S, Hertze J, Nagga K, Nilsson K, Nilsson C, Swedish Bio FSG, et al. Increased blood-brain barrier permeability is associated with dementia and diabetes but not amyloid pathology or APOE genotype. *Neurobiol Aging*. 2017;51:104-12.
30. Tibbling G, Link H, Ohman S. Principles of albumin and IgG analyses in neurological disorders. I. Establishment of reference values. *Scand J Clin Lab Invest*. 1977;37(5):385-90.

31. Farkas E, Luiten PG. Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol.* 2001;64(6):575-611.
32. Desai BS, Schneider JA, Li JL, Carvey PM, Hendey B. Evidence of angiogenic vessels in Alzheimer's disease. *J Neural Transm.* 2009;116(5):587-97.
33. Maia LF, Mackenzie IR, Feldman HH. Clinical phenotypes of Cerebral Amyloid Angiopathy. *J Neurol Sci.* 2007;257(1-2):23-30.
34. Jellinger KA. Alzheimer disease and cerebrovascular pathology: an update. *J Neural Transm (Vienna).* 2002;109(5-6):813-36.
35. Thal DR, Ghebremedhin E, Rub U, Yamaguchi H, Del Tredici K, Braak H. Two types of sporadic cerebral amyloid angiopathy. *J Neuropathol Exp Neurol.* 2002;61(3):282-93.
36. Roher AE, Debbins JP, Malek-Ahmadi M, Chen K, Pipe JG, Maze S, et al. Cerebral blood flow in Alzheimer's disease. *Vasc Health Risk Manag.* 2012;8:599-611.
37. Vernooij MW, van der Lugt A, Ikram MA, Wielopolski PA, Niessen WJ, Hofman A, et al. Prevalence and risk factors of cerebral microbleeds: the Rotterdam Scan Study. *Neurology.* 2008;70(14):1208-14.
38. Sveinbjornsdottir S, Sigurdsson S, Aspelund T, Kjartansson O, Eiriksdottir G, Valtysdottir B, et al. Cerebral microbleeds in the population based AGES-Reykjavik study: prevalence and location. *J Neurol Neurosurg Psychiatry.* 2008;79(9):1002-6.
39. Goos JD, Kester MI, Barkhof F, Klein M, Blankenstein MA, Scheltens P, et al. Patients with Alzheimer disease with multiple microbleeds: relation with cerebrospinal fluid biomarkers and cognition. *Stroke.* 2009;40(11):3455-60.
40. Reijmer YD, van Veluw SJ, Greenberg SM. Ischemic brain injury in cerebral amyloid angiopathy. *J Cereb Blood Flow Metab.* 2016;36(1):40-54.
41. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res.* 1975;12(3):189-98.
42. Nasreddine ZS, Phillips NA, Bedirian V, Charbonneau S, Whitehead V, Collin I, et al. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *J Am Geriatr Soc.* 2005;53(4):695-9.
43. Shulman KI. Clock-drawing: is it the ideal cognitive screening test? *Int J Geriatr Psychiatry.* 2000;15(6):548-61.
44. Park M, Moon WJ. Structural MR Imaging in the Diagnosis of Alzheimer's Disease and Other Neurodegenerative Dementia: Current Imaging Approach and Future Perspectives. *Korean J Radiol.* 2016;17(6):827-45.
45. Blennow K. A Review of Fluid Biomarkers for Alzheimer's Disease: Moving from CSF to Blood. *Neurol Ther.* 2017;6(Suppl 1):15-24.
46. Wu YY, Hsu JL, Wang HC, Wu SJ, Hong CJ, Cheng IH. Alterations of the Neuroinflammatory Markers IL-6 and TRAIL in Alzheimer's Disease. *Dement Geriatr Cogn Dis Extra.* 2015;5(3):424-34.
47. Craig-Schapiro R, Perrin RJ, Roe CM, Xiong C, Carter D, Cairns NJ, et al. YKL-40: a novel prognostic fluid biomarker for preclinical Alzheimer's disease. *Biological psychiatry.* 2010;68(10):903-12.

48. Muszynski P, Groblewska M, Kulczynska-Przybik A, Kulakowska A, Mroczko B. YKL-40 as a Potential Biomarker and a Possible Target in Therapeutic Strategies of Alzheimer's Disease. *Curr Neuropharmacol*. 2017;15(6):906-17.
49. Yaffe K, Kanaya A, Lindquist K, Simonsick EM, Harris T, Shorr RI, et al. The metabolic syndrome, inflammation, and risk of cognitive decline. *JAMA*. 2004;292(18):2237-42.
50. Portelius E, Zetterberg H, Skillback T, Tornqvist U, Andreasson U, Trojanowski JQ, et al. Cerebrospinal fluid neurogranin: relation to cognition and neurodegeneration in Alzheimer's disease. *Brain*. 2015;138(Pt 11):3373-85.
51. Kang JH, Korecka M, Figurski MJ, Toledo JB, Blennow K, Zetterberg H, et al. The Alzheimer's Disease Neuroimaging Initiative 2 Biomarker Core: A review of progress and plans. *Alzheimers Dement*. 2015;11(7):772-91.
52. Mattsson N, Insel PS, Palmqvist S, Portelius E, Zetterberg H, Weiner M, et al. Cerebrospinal fluid tau, neurogranin, and neurofilament light in Alzheimer's disease. *EMBO Mol Med*. 2016;8(10):1184-96.
53. Stomrud E, Bjorkqvist M, Janciauskiene S, Minthon L, Hansson O. Alterations of matrix metalloproteinases in the healthy elderly with increased risk of prodromal Alzheimer's disease. *Alzheimers Res Ther*. 2010;2(3):20.
54. Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. *Arch Neurol*. 1998;55(11):1449-55.
55. Leung YY, Toledo JB, Nefedov A, Polikar R, Raghavan N, Xie SX, et al. Identifying amyloid pathology-related cerebrospinal fluid biomarkers for Alzheimer's disease in a multicohort study. *Alzheimers Dement (Amst)*. 2015;1(3):339-48.
56. Nagga K, Hansson O, van Westen D, Minthon L, Wennstrom M. Increased levels of hyaluronic acid in cerebrospinal fluid in patients with vascular dementia. *J Alzheimers Dis*. 2014;42(4):1435-41.
57. Nielsen HM, Palmqvist S, Minthon L, Londos E, Wennstrom M. Gender-dependent levels of hyaluronic acid in cerebrospinal fluid of patients with neurodegenerative dementia. *Curr Alzheimer Res*. 2012;9(3):257-66.
58. Koronyo-Hamaoui M, Koronyo Y, Ljubimov AV, Miller CA, Ko MK, Black KL, et al. Identification of amyloid plaques in retinas from Alzheimer's patients and noninvasive in vivo optical imaging of retinal plaques in a mouse model. *Neuroimage*. 2011;54 Suppl 1:S204-17.
59. Loffler KU, Edward DP, Tso MO. Immunoreactivity against tau, amyloid precursor protein, and beta-amyloid in the human retina. *Invest Ophthalmol Vis Sci*. 1995;36(1):24-31.
60. Johnson LV, Leitner WP, Rivest AJ, Staples MK, Radeke MJ, Anderson DH. The Alzheimer's A beta -peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2002;99(18):11830-5.
61. Frost S, Kanagasalingam Y, Sohrabi H, Vignarajan J, Bourgeat P, Salvado O, et al. Retinal vascular biomarkers for early detection and monitoring of Alzheimer's disease. *Transl Psychiatry*. 2013;3:e233.

62. Kelleher RJ, 3rd, Shen J. Presenilin-1 mutations and Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(4):629-31.
63. Welander H, Franberg J, Graff C, Sundstrom E, Winblad B, Tjernberg LO. Abeta43 is more frequent than Abeta40 in amyloid plaque cores from Alzheimer disease brains. *Journal of neurochemistry*. 2009;110(2):697-706.
64. Chavez-Gutierrez L, Bammens L, Benilova I, Vandersteen A, Benurwar M, Borgers M, et al. The mechanism of gamma-Secretase dysfunction in familial Alzheimer disease. *EMBO J*. 2012;31(10):2261-74.
65. Rusanen M, Rovio S, Ngandu T, Nissinen A, Tuomilehto J, Soininen H, et al. Midlife smoking, apolipoprotein E and risk of dementia and Alzheimer's disease: a population-based cardiovascular risk factors, aging and dementia study. *Dement Geriatr Cogn Disord*. 2010;30(3):277-84.
66. Laitinen MH, Ngandu T, Rovio S, Helkala EL, Uusitalo U, Viitanen M, et al. Fat intake at midlife and risk of dementia and Alzheimer's disease: a population-based study. *Dement Geriatr Cogn Disord*. 2006;22(1):99-107.
67. Eskelinen MH, Ngandu T, Tuomilehto J, Soininen H, Kivipelto M. Midlife healthy-diet index and late-life dementia and Alzheimer's disease. *Dement Geriatr Cogn Dis Extra*. 2011;1(1):103-12.
68. Rovio S, Kareholt I, Helkala EL, Viitanen M, Winblad B, Tuomilehto J, et al. Leisure-time physical activity at midlife and the risk of dementia and Alzheimer's disease. *Lancet Neurol*. 2005;4(11):705-11.
69. Tolppanen AM, Solomon A, Kulmala J, Kareholt I, Ngandu T, Rusanen M, et al. Leisure-time physical activity from mid- to late life, body mass index, and risk of dementia. *Alzheimers Dement*. 2015;11(4):434-43 e6.
70. Flicker L. Modifiable lifestyle risk factors for Alzheimer's disease. *Journal of Alzheimer's disease : JAD*. 2010;20(3):803-11.
71. Larsson SC, Traylor M, Malik R, Dichgans M, Burgess S, Markus HS, et al. Modifiable pathways in Alzheimer's disease: Mendelian randomisation analysis. *BMJ*. 2017;359:j5375.
72. Ott A, Breteler MM, van Harskamp F, Claus JJ, van der Cammen TJ, Grobbee DE, et al. Prevalence of Alzheimer's disease and vascular dementia: association with education. The Rotterdam study. *BMJ*. 1995;310(6985):970-3.
73. Lee CY, Landreth GE. The role of microglia in amyloid clearance from the AD brain. *J Neural Transm (Vienna)*. 2010;117(8):949-60.
74. Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nat Rev Neurol*. 2010;6(4):193-201.
75. Swardfager W, Lancot K, Rothenburg L, Wong A, Cappell J, Herrmann N. A meta-analysis of cytokines in Alzheimer's disease. *Biological psychiatry*. 2010;68(10):930-41.
76. in 't Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, et al. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *The New England journal of medicine*. 2001;345(21):1515-21.

77. Cornelius C, Fastbom J, Winblad B, Viitanen M. Aspirin, NSAIDs, risk of dementia, and influence of the apolipoprotein E epsilon 4 allele in an elderly population. *Neuroepidemiology*. 2004;23(3):135-43.
78. Etminan M, Gill S, Samii A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. *BMJ*. 2003;327(7407):128.
79. Holtzman DM, Bales KR, Tenkova T, Fagan AM, Parsadanian M, Sartorius LJ, et al. Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97(6):2892-7.
80. Strittmatter WJ, Weisgraber KH, Huang DY, Dong LM, Salvesen GS, Pericak-Vance M, et al. Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90(17):8098-102.
81. Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, et al. Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *The Journal of clinical investigation*. 2000;106(12):1489-99.
82. Hayden KM, Zandi PP, Khachaturian AS, Szekely CA, Fotuhi M, Norton MC, et al. Does NSAID use modify cognitive trajectories in the elderly? The Cache County study. *Neurology*. 2007;69(3):275-82.
83. Hofman A, Ott A, Breteler MM, Bots ML, Slioter AJ, van Harskamp F, et al. Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet*. 1997;349(9046):151-4.
84. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology*. 1999;53(9):1937-42.
85. Peila R, Rodriguez BL, Launer LJ, Honolulu-Asia Aging S. Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study. *Diabetes*. 2002;51(4):1256-62.
86. Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Arch Neurol*. 2004;61(5):661-6.
87. Luchsinger JA, Reitz C, Honig LS, Tang MX, Shea S, Mayeux R. Aggregation of vascular risk factors and risk of incident Alzheimer disease. *Neurology*. 2005;65(4):545-51.
88. Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol*. 2006;5(1):64-74.
89. Irie F, Fitzpatrick AL, Lopez OL, Kuller LH, Peila R, Newman AB, et al. Enhanced risk for Alzheimer disease in persons with type 2 diabetes and APOE epsilon4: the Cardiovascular Health Study Cognition Study. *Arch Neurol*. 2008;65(1):89-93.

90. Biessels GJ, Kappelle LJ. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? *Biochem Soc Trans.* 2005;33(Pt 5):1041-4.
91. Yoshitake T, Kiyohara Y, Kato I, Ohmura T, Iwamoto H, Nakayama K, et al. Incidence and risk factors of vascular dementia and Alzheimer's disease in a defined elderly Japanese population: the Hisayama Study. *Neurology.* 1995;45(6):1161-8.
92. Exalto LG, Biessels GJ, Karter AJ, Huang ES, Katon WJ, Minkoff JR, et al. Risk score for prediction of 10 year dementia risk in individuals with type 2 diabetes: a cohort study. *Lancet Diabetes Endocrinol.* 2013;1(3):183-90.
93. Iadecola C. The pathobiology of vascular dementia. *Neuron.* 2013;80(4):844-66.
94. Rouget. Mémoire sur le développement, la structure et les propriétés physiologiques des capillaires sanguins et lymphatiques. *Arch Physiol Norm Path* 1873;5:603-63
95. Eberth. *Handbuch der Lehre von der Gewegen des Menschen und der Tiere.* Leipzig: Engelmann. 1871;1.
96. Zimmermann. Der feinere Bau der Blutkapillaren. *Anat Entwicklungsgesch* 1923.
97. Winkler EA, Bell RD, Zlokovic BV. Central nervous system pericytes in health and disease. *Nat Neurosci.* 2011;14(11):1398-405.
98. Attwell D, Mishra A, Hall CN, O'Farrell FM, Dalkara T. What is a pericyte? *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.* 2016;36(2):451-5.
99. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Developmental cell.* 2011;21(2):193-215.
100. Sims DE. The pericyte--a review. *Tissue Cell.* 1986;18(2):153-74.
101. Frank RN, Turczyn TJ, Das A. Pericyte coverage of retinal and cerebral capillaries. *Invest Ophthalmol Vis Sci.* 1990;31(6):999-1007.
102. Smyth LCD, Rustenhoven J, Scotter EL, Schweder P, Faull RLM, Park TIH, et al. Markers for human brain pericytes and smooth muscle cells. *J Chem Neuroanat.* 2018;92:48-60.
103. Yousif LF, Di Russo J, Sorokin L. Laminin isoforms in endothelial and perivascular basement membranes. *Cell Adh Migr.* 2013;7(1):101-10.
104. Nehls V, Drenckhahn D. Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J Cell Biol.* 1991;113(1):147-54.
105. Trost A, Schroedl F, Lange S, Rivera FJ, Tempfer H, Korntrner S, et al. Neural crest origin of retinal and choroidal pericytes. *Invest Ophthalmol Vis Sci.* 2013;54(13):7910-21.
106. Trost A, Lange S, Schroedl F, Bruckner D, Motloch KA, Bogner B, et al. Brain and Retinal Pericytes: Origin, Function and Role. *Front Cell Neurosci.* 2016;10:20.
107. Kunz J, Krause D, Kremer M, Dermietzel R. The 140-kDa protein of blood-brain barrier-associated pericytes is identical to aminopeptidase N. *Journal of neurochemistry.* 1994;62(6):2375-86.

108. Schlingemann RO, Rietveld FJ, de Waal RM, Ferrone S, Ruiter DJ. Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *Am J Pathol.* 1990;136(6):1393-405.
109. Cho H, Kozasa T, Bondjers C, Betsholtz C, Kehrl JH. Pericyte-specific expression of Rgs5: implications for PDGF and EDG receptor signaling during vascular maturation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2003;17(3):440-2.
110. MacFadyen J, Savage K, Wienke D, Isacke CM. Endosialin is expressed on stromal fibroblasts and CNS pericytes in mouse embryos and is downregulated during development. *Gene Expr Patterns.* 2007;7(3):363-9.
111. Nehls V, Denzer K, Drenckhahn D. Pericyte involvement in capillary sprouting during angiogenesis in situ. *Cell Tissue Res.* 1992;270(3):469-74.
112. Diaz-Flores L, Gutierrez R, Varela H, Rancel N, Valladares F. Microvascular pericytes: a review of their morphological and functional characteristics. *Histol Histopathol.* 1991;6(2):269-86.
113. Bondjers C, He L, Takemoto M, Norlin J, Asker N, Hellstrom M, et al. Microarray analysis of blood microvessels from PDGF-B and PDGF-Rbeta mutant mice identifies novel markers for brain pericytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(10):1703-5.
114. Davson H. Review lecture. The blood-brain barrier. *J Physiol.* 1976;255(1):1-28.
115. Daneman R, Prat A. The blood-brain barrier. *Cold Spring Harb Perspect Biol.* 2015;7(1):a020412.
116. Daneman R. The blood-brain barrier in health and disease. *Ann Neurol.* 2012;72(5):648-72.
117. Gastfriend BD, Palecek SP, Shusta EV. Modeling the blood-brain barrier: Beyond the endothelial cells. *Curr Opin Biomed Eng.* 2018;5:6-12.
118. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature.* 2010;468(7323):562-6.
119. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 1994;8(16):1875-87.
120. Soriano P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev.* 1994;8(16):1888-96.
121. Li F, Lan Y, Wang Y, Wang J, Yang G, Meng F, et al. Endothelial Smad4 maintains cerebrovascular integrity by activating N-cadherin through cooperation with Notch. *Developmental cell.* 2011;20(3):291-302.
122. Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, et al. Pericytes regulate the blood-brain barrier. *Nature.* 2010;468(7323):557-61.
123. Thomas WE. Brain macrophages: on the role of pericytes and perivascular cells. *Brain Res Brain Res Rev.* 1999;31(1):42-57.
124. Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, et al. Solutes, but not cells, drain from the brain parenchyma along basement membranes

- of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. *Neuropathol Appl Neurobiol.* 2008;34(2):131-44.
125. Diem AK, MacGregor Sharp M, Gatherer M, Bressloff NW, Carare RO, Richardson G. Arterial Pulsations cannot Drive Intramural Periarterial Drainage: Significance for Abeta Drainage. *Frontiers in neuroscience.* 2017;11:475.
 126. Cunha-Vaz J, Bernardes R, Lobo C. Blood-retinal barrier. *Eur J Ophthalmol.* 2011;21 Suppl 6:S3-9.
 127. Park DY, Lee J, Kim J, Kim K, Hong S, Han S, et al. Plastic roles of pericytes in the blood-retinal barrier. *Nat Commun.* 2017;8:15296.
 128. Simo R, Villarroel M, Corraliza L, Hernandez C, Garcia-Ramirez M. The retinal pigment epithelium: something more than a constituent of the blood-retinal barrier--implications for the pathogenesis of diabetic retinopathy. *J Biomed Biotechnol.* 2010;2010:190724.
 129. Diaz-Coranguéz M, Ramos C, Antonetti DA. The inner blood-retinal barrier: Cellular basis and development. *Vision Res.* 2017;139:123-37.
 130. Frey T, Antonetti DA. Alterations to the blood-retinal barrier in diabetes: cytokines and reactive oxygen species. *Antioxidants & redox signaling.* 2011;15(5):1271-84.
 131. Beltramo E, Porta M. Pericyte loss in diabetic retinopathy: mechanisms and consequences. *Curr Med Chem.* 2013;20(26):3218-25.
 132. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol.* 2005;7(4):452-64.
 133. Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA. An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proceedings of the National Academy of Sciences of the United States of America.* 1989;86(12):4544-8.
 134. Risau W. Mechanisms of angiogenesis. *Nature.* 1997;386(6626):671-4.
 135. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res.* 2003;314(1):15-23.
 136. Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol.* 1998;141(3):805-14.
 137. Gaengel K, Genove G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol.* 2009;29(5):630-8.
 138. Shih AH, Holland EC. Platelet-derived growth factor (PDGF) and glial tumorigenesis. *Cancer Lett.* 2006;232(2):139-47.
 139. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development.* 1998;125(9):1591-8.
 140. Antonelli-Orlidge A, Smith SR, D'Amore PA. Influence of pericytes on capillary endothelial cell growth. *Am Rev Respir Dis.* 1989;140(4):1129-31.

141. Ozen I, Deierborg T, Miharada K, Padel T, Englund E, Genove G, et al. Brain pericytes acquire a microglial phenotype after stroke. *Acta Neuropathol.* 2014;128(3):381-96.
142. Gaceb A, Barbariga M, Ozen I, Paul G. The pericyte secretome: Potential impact on regeneration. *Biochimie.* 2018.
143. Sato Y. Activation of latent TGF-beta at the vascular wall--roles of endothelial cells and mural pericytes or smooth muscle cells. *J Atheroscler Thromb.* 1995;2(1):24-9.
144. Ribatti D, Nico B, Crivellato E. The role of pericytes in angiogenesis. *Int J Dev Biol.* 2011;55(3):261-8.
145. Thanabalasundaram G, Schneidewind J, Pieper C, Galla HJ. The impact of pericytes on the blood-brain barrier integrity depends critically on the pericyte differentiation stage. *Int J Biochem Cell Biol.* 2011;43(9):1284-93.
146. Takahashi Y, Maki T, Liang AC, Itoh K, Lok J, Osumi N, et al. p38 MAP kinase mediates transforming-growth factor-beta1-induced upregulation of matrix metalloproteinase-9 but not -2 in human brain pericytes. *Brain research.* 2014;1593:1-8.
147. Weekman EM, Wilcock DM. Matrix Metalloproteinase in Blood-Brain Barrier Breakdown in Dementia. *Journal of Alzheimer's disease : JAD.* 2016;49(4):893-903.
148. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.* 2007;27(4):697-709.
149. Takata F, Dohgu S, Matsumoto J, Takahashi H, Machida T, Wakigawa T, et al. Brain pericytes among cells constituting the blood-brain barrier are highly sensitive to tumor necrosis factor-alpha, releasing matrix metalloproteinase-9 and migrating in vitro. *J Neuroinflammation.* 2011;8:106.
150. Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z, et al. Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature.* 2012;485(7399):512-6.
151. You WK, Yotsumoto F, Sakimura K, Adams RH, Stallcup WB. NG2 proteoglycan promotes tumor vascularization via integrin-dependent effects on pericyte function. *Angiogenesis.* 2014;17(1):61-76.
152. Maus F, Sakry D, Biname F, Karraam K, Rajalingam K, Watts C, et al. The NG2 Proteoglycan Protects Oligodendrocyte Precursor Cells against Oxidative Stress via Interaction with OMI/HtrA2. *PLoS One.* 2015;10(9):e0137311.
153. Sakry D, Neitz A, Singh J, Frischknecht R, Marongiu D, Biname F, et al. Oligodendrocyte precursor cells modulate the neuronal network by activity-dependent ectodomain cleavage of glial NG2. *PLoS Biol.* 2014;12(11):e1001993.
154. Nielsen H, Ek D, Avdic U, Orbjorn C, Hansson O, Bank TNB, et al. NG2 cells, a new trail for Alzheimer's disease mechanisms? *Acta Neuropathologica Communications.* 2013;1(1):7.
155. Baloyannis SJ, Baloyannis IS. The vascular factor in Alzheimer's disease: a study in Golgi technique and electron microscopy. *J Neurol Sci.* 2012;322(1-2):117-21.

156. Keable A, Fenna K, Yuen HM, Johnston DA, Smyth NR, Smith C, et al. Deposition of amyloid beta in the walls of human leptomeningeal arteries in relation to perivascular drainage pathways in cerebral amyloid angiopathy. *Biochim Biophys Acta*. 2016;1862(5):1037-46.
157. Weller RO, Hawkes CA, Carare RO, Hardy J. Does the difference between PART and Alzheimer's disease lie in the age-related changes in cerebral arteries that trigger the accumulation of Abeta and propagation of tau? *Acta neuropathologica*. 2015;129(5):763-6.
158. Sagare AP, Bell RD, Zhao Z, Ma Q, Winkler EA, Ramanathan A, et al. Pericyte loss influences Alzheimer-like neurodegeneration in mice. *Nat Commun*. 2013;4:2932.
159. Young-Pearse TL, Bai J, Chang R, Zheng JB, LoTurco JJ, Selkoe DJ. A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007;27(52):14459-69.
160. Löffler J, Huber G. Beta-amyloid precursor protein isoforms in various rat brain regions and during brain development. *Journal of neurochemistry*. 1992;59(4):1316-24.
161. Magara F, Muller U, Li ZW, Lipp HP, Weissmann C, Stagljar M, et al. Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the beta-amyloid-precursor protein. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(8):4656-61.
162. Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, O'Dowd G, et al. Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience*. 1999;90(1):1-13.
163. Tyan SH, Shih AY, Walsh JJ, Maruyama H, Sarsoza F, Ku L, et al. Amyloid precursor protein (APP) regulates synaptic structure and function. *Mol Cell Neurosci*. 2012;51(1-2):43-52.
164. Smith RP, Higuchi DA, Broze GJ, Jr. Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein. *Science*. 1990;248(4959):1126-8.
165. Chasseigneaux S, Allinquant B. Functions of Abeta, sAPPalpha and sAPPbeta : similarities and differences. *Journal of neurochemistry*. 2012;120 Suppl 1:99-108.
166. Meziane H, Dodart JC, Mathis C, Little S, Clemens J, Paul SM, et al. Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnesic mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95(21):12683-8.
167. Taylor CJ, Ireland DR, Ballagh I, Bourne K, Marechal NM, Turner PR, et al. Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. *Neurobiol Dis*. 2008;31(2):250-60.
168. Wisniewski HM, Frackowiak J. Commentary to: Differences between the pathogenesis of senile plaques and congophilic angiopathy in Alzheimer disease. (*J Neuropathol Exp Neurol* 1997; 56:751-61). *J Neuropathol Exp Neurol*. 1998;57(1):96-8.

169. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med.* 2012;2(5):a006270.
170. Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, et al. Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2008;28(53):14537-45.
171. Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, et al. BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2005;25(50):11693-709.
172. Fonseca MB, Sola S, Xavier JM, Dionisio PA, Rodrigues CM. Amyloid beta peptides promote autophagy-dependent differentiation of mouse neural stem cells: Abeta-mediated neural differentiation. *Molecular neurobiology.* 2013;48(3):829-40.
173. Giuffrida ML, Caraci F, Pignataro B, Cataldo S, De Bona P, Bruno V, et al. Beta-amyloid monomers are neuroprotective. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2009;29(34):10582-7.
174. Wong CW, Quaranta V, Glenner GG. Neuritic plaques and cerebrovascular amyloid in Alzheimer disease are antigenically related. *Proc Natl Acad Sci U S A.* 1985;82(24):8729-32.
175. Walsh DM, Lomakin A, Benedek GB, Condron MM, Teplow DB. Amyloid beta-protein fibrillogenesis. Detection of a protofibrillar intermediate. *The Journal of biological chemistry.* 1997;272(35):22364-72.
176. Gitter BD, Cox LM, Rydel RE, May PC. Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells. *Proc Natl Acad Sci U S A.* 1995;92(23):10738-41.
177. Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem.* 2002;277(35):32046-53.
178. Gu L, Guo Z. Alzheimer's Abeta42 and Abeta40 peptides form interlaced amyloid fibrils. *Journal of neurochemistry.* 2013;126(3):305-11.
179. Plant LD, Boyle JP, Smith IF, Peers C, Pearson HA. The production of amyloid beta peptide is a critical requirement for the viability of central neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2003;23(13):5531-5.
180. Wilhelmus MM, Otte-Holler I, van Triel JJ, Veerhuis R, Maat-Schieman ML, Bu G, et al. Lipoprotein receptor-related protein-1 mediates amyloid-beta-mediated cell death of cerebrovascular cells. *Am J Pathol.* 2007;171(6):1989-99.
181. Verbeek MM, de Waal RM, Schipper JJ, Van Nostrand WE. Rapid degeneration of cultured human brain pericytes by amyloid beta protein. *J Neurochem.* 1997;68(3):1135-41.
182. Rensink AA, Verbeek MM, Otte-Holler I, ten Donkelaar HT, de Waal RM, Kremer B. Inhibition of amyloid-beta-induced cell death in human brain pericytes in vitro. *Brain Res.* 2002;952(1):111-21.

183. Winkler EA, Sagare AP, Zlokovic BV. The pericyte: a forgotten cell type with important implications for Alzheimer's disease? *Brain Pathol.* 2014;24(4):371-86.
184. Nortley R, Mishra A, Jaunmuktane Z, Kyrargyri V, Madry C, Gong H, et al. Amyloid beta oligomers constrict human capillaries in Alzheimer's disease via signalling to pericytes. *bioRxiv.* 2018.
185. Westermark P, Andersson A, Westermark GT. Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. *Physiol Rev.* 2011;91(3):795-826.
186. Jackson K, Barisone GA, Diaz E, Jin LW, DeCarli C, Despa F. Amylin deposition in the brain: A second amyloid in Alzheimer disease? *Ann Neurol.* 2013;74(4):517-26.
187. Oskarsson ME, Paulsson JF, Schultz SW, Ingelsson M, Westermark P, Westermark GT. In vivo seeding and cross-seeding of localized amyloidosis: a molecular link between type 2 diabetes and Alzheimer disease. *Am J Pathol.* 2015;185(3):834-46.
188. Butler PC, Chou J, Carter WB, Wang YN, Bu BH, Chang D, et al. Effects of meal ingestion on plasma amylin concentration in NIDDM and nondiabetic humans. *Diabetes.* 1990;39(6):752-6.
189. Balasubramaniam A, Renugopalakrishnan V, Stein M, Fischer JE, Chance WT. Syntheses, structures and anorectic effects of human and rat amylin. *Peptides.* 1991;12(5):919-24.
190. Li Z, Kelly L, Heiman M, Greengard P, Friedman JM. Hypothalamic Amylin Acts in Concert with Leptin to Regulate Food Intake. *Cell Metab.* 2015;22(6):1059-67.
191. Braegger FE, Asarian L, Dahl K, Lutz TA, Boyle CN. The role of the area postrema in the anorectic effects of amylin and salmon calcitonin: behavioral and neuronal phenotyping. *The European journal of neuroscience.* 2014;40(7):3055-66.
192. Szabo ER, Cservenak M, Dobolyi A. Amylin is a novel neuropeptide with potential maternal functions in the rat. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2012;26(1):272-81.
193. Srodulski S, Sharma S, Bachstetter AB, Brelsfoard JM, Pascual C, Xie XS, et al. Neuroinflammation and neurologic deficits in diabetes linked to brain accumulation of amylin. *Molecular neurodegeneration.* 2014;9:30.
194. Jeong HR, An SS. Causative factors for formation of toxic islet amyloid polypeptide oligomer in type 2 diabetes mellitus. *Clin Interv Aging.* 2015;10:1873-9.
195. Dodson G, Steiner D. The role of assembly in insulin's biosynthesis. *Curr Opin Struct Biol.* 1998;8(2):189-94.
196. Westermark P, Li ZC, Westermark GT, Leckstrom A, Steiner DF. Effects of beta cell granule components on human islet amyloid polypeptide fibril formation. *FEBS Lett.* 1996;379(3):203-6.
197. Kahn SE, D'Alessio DA, Schwartz MW, Fujimoto WY, Ensinnck JW, Taborsky GJ, Jr., et al. Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. *Diabetes.* 1990;39(5):634-8.
198. Ludvik B, Kautzky-Willer A, Prager R, Thomaseth K, Pacini G. Amylin: history and overview. *Diabet Med.* 1997;14 Suppl 2:S9-13.
199. Gedulin BR, Rink TJ, Young AA. Dose-response for glucagonostatic effect of amylin in rats. *Metabolism.* 1997;46(1):67-70.

200. Lutz TA, Mollet A, Rushing PA, Riediger T, Scharrer E. The anorectic effect of a chronic peripheral infusion of amylin is abolished in area postrema/nucleus of the solitary tract (AP/NTS) lesioned rats. *Int J Obes Relat Metab Disord*. 2001;25(7):1005-11.
201. Banks WA, Kastin AJ, Maness LM, Huang W, Jaspan JB. Permeability of the blood-brain barrier to amylin. *Life sciences*. 1995;57(22):1993-2001.
202. Hull RL, Westermark GT, Westermark P, Kahn SE. Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. *J Clin Endocrinol Metab*. 2004;89(8):3629-43.
203. Gilead S, Wolfenson H, Gazit E. Molecular mapping of the recognition interface between the islet amyloid polypeptide and insulin. *Angew Chem Int Ed Engl*. 2006;45(39):6476-80.
204. Despa S, Margulies KB, Chen L, Knowlton AA, Havel PJ, Taegtmeier H, et al. Hyperamylinemia contributes to cardiac dysfunction in obesity and diabetes: a study in humans and rats. *Circ Res*. 2012;110(4):598-608.
205. Gong W, Liu ZH, Zeng CH, Peng A, Chen HP, Zhou H, et al. Amylin deposition in the kidney of patients with diabetic nephropathy. *Kidney Int*. 2007;72(2):213-8.
206. Lorenzo A, Razzaboni B, Weir GC, Yankner BA. Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature*. 1994;368(6473):756-60.
207. O'Brien TD, Butler PC, Westermark P, Johnson KH. Islet amyloid polypeptide: a review of its biology and potential roles in the pathogenesis of diabetes mellitus. *Vet Pathol*. 1993;30(4):317-32.
208. Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJ. The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells. *FEBS J*. 2006;273(15):3614-24.
209. Green J, Goldsberry C, Mini T, Sunderji S, Frey P, Kistler J, et al. Full-length rat amylin forms fibrils following substitution of single residues from human amylin. *J Mol Biol*. 2003;326(4):1147-56.
210. Westermark P, Engstrom U, Johnson KH, Westermark GT, Betsholtz C. Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87(13):5036-40.
211. Pillay K, Govender P. Amylin uncovered: a review on the polypeptide responsible for type II diabetes. *BioMed research international*. 2013;2013:826706.
212. Wu C, Shea JE. Structural similarities and differences between amyloidogenic and non-amyloidogenic islet amyloid polypeptide (IAPP) sequences and implications for the dual physiological and pathological activities of these peptides. *PLoS Comput Biol*. 2013;9(8):e1003211.
213. Su Y, Chang PT. Acidic pH promotes the formation of toxic fibrils from beta-amyloid peptide. *Brain Res*. 2001;893(1-2):287-91.
214. Klement K, Wieligmann K, Meinhardt J, Hortschansky P, Richter W, Fandrich M. Effect of different salt ions on the propensity of aggregation and on the structure of Alzheimer's abeta(1-40) amyloid fibrils. *J Mol Biol*. 2007;373(5):1321-33.

215. Abedini A, Raleigh DP. The role of His-18 in amyloid formation by human islet amyloid polypeptide. *Biochemistry*. 2005;44(49):16284-91.
216. Jha S, Snell JM, Sheftic SR, Patil SM, Daniels SB, Kolling FW, et al. pH dependence of amylin fibrillization. *Biochemistry*. 2014;53(2):300-10.
217. Clark A, Nilsson MR. Islet amyloid: a complication of islet dysfunction or an aetiological factor in Type 2 diabetes? *Diabetologia*. 2004;47(2):157-69.
218. Lim YA, Ittner LM, Lim YL, Gotz J. Human but not rat amylin shares neurotoxic properties with Abeta42 in long-term hippocampal and cortical cultures. *FEBS Lett*. 2008;582(15):2188-94.
219. Fu W, Ruangkittisakul A, MacTavish D, Shi JY, Ballanyi K, Jhamandas JH. Amyloid beta (Abeta) peptide directly activates amylin-3 receptor subtype by triggering multiple intracellular signaling pathways. *J Biol Chem*. 2012;287(22):18820-30.
220. Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, et al. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *J Biol Chem*. 1998;273(49):32730-8.
221. Qiu WQ, Wallack M, Dean M, Liebson E, Mwamburi M, Zhu H. Association between amylin and amyloid-beta peptides in plasma in the context of apolipoprotein E4 allele. *PLoS One*. 2014;9(2):e88063.
222. Moreno-Gonzalez I, Edwards Iii G, Salvadores N, Shahnawaz M, Diaz-Espinoza R, Soto C. Molecular interaction between type 2 diabetes and Alzheimer's disease through cross-seeding of protein misfolding. *Mol Psychiatry*. 2017;22(9):1327-34.
223. Mukherjee A, Morales-Scheihing D, Salvadores N, Moreno-Gonzalez I, Gonzalez C, Taylor-Prese K, et al. Induction of IAPP amyloid deposition and associated diabetic abnormalities by a prion-like mechanism. *The Journal of experimental medicine*. 2017;214(9):2591-610.
224. Andreetto E, Yan LM, Caporale A, Kapurniotu A. Dissecting the role of single regions of an IAPP mimic and IAPP in inhibition of Abeta40 amyloid formation and cytotoxicity. *Chembiochem*. 2011;12(9):1313-22.
225. Andreetto E, Yan LM, Taterek-Nossol M, Velkova A, Frank R, Kapurniotu A. Identification of hot regions of the Abeta-IAPP interaction interface as high-affinity binding sites in both cross- and self-association. *Angew Chem Int Ed Engl*. 2010;49(17):3081-5.
226. Zhang M, Hu R, Chen H, Gong X, Zhou F, Zhang L, et al. Polymorphic Associations and Structures of the Cross-Seeding of Abeta1-42 and hIAPP1-37 Polypeptides. *J Chem Inf Model*. 2015;55(8):1628-39.
227. Yan LM, Velkova A, Kapurniotu A. Molecular characterization of the hetero-assembly of beta-amyloid peptide with islet amyloid polypeptide. *Curr Pharm Des*. 2014;20(8):1182-91.
228. Zhu H, Wang X, Wallack M, Li H, Carreras I, Dedeoglu A, et al. Intraperitoneal injection of the pancreatic peptide amylin potently reduces behavioral impairment and brain amyloid pathology in murine models of Alzheimer's disease. *Mol Psychiatry*. 2014.

229. Adler BL, Yarchoan M, Hwang HM, Louneva N, Blair JA, Palm R, et al. Neuroprotective effects of the amylin analogue pramlintide on Alzheimer's disease pathogenesis and cognition. *Neurobiol Aging*. 2014;35(4):793-801.
230. Qiu WQ, Au R, Zhu H, Wallack M, Liebson E, Li H, et al. Positive association between plasma amylin and cognition in a homebound elderly population. *Journal of Alzheimer's disease : JAD*. 2014;42(2):555-63.
231. Qiu WQ, Zhu H. Amylin and its analogs: a friend or foe for the treatment of Alzheimer's disease? *Front Aging Neurosci*. 2014;6:186.
232. Mohamed LA, Zhu H, Mousa YM, Wang E, Qiu WQ, Kaddoumi A. Amylin Enhances Amyloid-beta Peptide Brain to Blood Efflux Across the Blood-Brain Barrier. *Journal of Alzheimer's disease : JAD*. 2017;56(3):1087-99.
233. Fu W, Vukojevic V, Patel A, Soudy R, MacTavish D, Westaway D, et al. Role of microglial amylin receptors in mediating beta amyloid (A β)-induced inflammation. *Journal of neuroinflammation*. 2017;14(1):199.
234. Conner AC, Simms J, Hay DL, Mahmoud K, Howitt SG, Wheatley M, et al. Heterodimers and family-B GPCRs: RAMPs, CGRP and adrenomedullin. *Biochem Soc Trans*. 2004;32(Pt 5):843-6.
235. Kis B, Kaiya H, Nishi R, Deli MA, Abraham CS, Yanagita T, et al. Cerebral endothelial cells are a major source of adrenomedullin. *J Neuroendocrinol*. 2002;14(4):283-93.
236. Abedini A, Cao P, Plesner A, Zhang J, He M, Derk J, et al. RAGE binds preamyloid IAPP intermediates and mediates pancreatic beta cell proteotoxicity. *J Clin Invest*. 2018;128(2):682-98.
237. El Assar M, Angulo J, Santos-Ruiz M, Moreno P, Novials A, Villanueva-Penacarrillo ML, et al. Differential effect of amylin on endothelial-dependent vasodilation in mesenteric arteries from control and insulin resistant rats. *PLoS One*. 2015;10(3):e0120479.
238. Lindhagen-Persson M, Brannstrom K, Vestling M, Steinitz M, Olofsson A. Amyloid-beta oligomer specificity mediated by the IgM isotype--implications for a specific protective mechanism exerted by endogenous auto-antibodies. *PloS one*. 2010;5(11):e13928.
239. Chan SH, Chan JY. Oxidative stress in carotid body contributes to enhanced chemoreflex in heart failure: focus on "Elevated mitochondrial superoxide contributes to enhanced chemoreflex in heart failure rabbits". *Am J Physiol Regul Integr Comp Physiol*. 2010;298(2):R301-2.
240. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol*. 1984;133(4):1710-5.
241. Stapor PC, Sweat RS, Dashti DC, Betancourt AM, Murfee WL. Pericyte dynamics during angiogenesis: new insights from new identities. *J Vasc Res*. 2014;51(3):163-74.
242. Liu H, Shubayev VI. Matrix metalloproteinase-9 controls proliferation of NG2+ progenitor cells immediately after spinal cord injury. *Exp Neurol*. 2011;231(2):236-46.

243. Loo DT, Copani A, Pike CJ, Whitemore ER, Walencewicz AJ, Cotman CW. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci U S A*. 1993;90(17):7951-5.
244. Watt JA, Pike CJ, Walencewicz-Wasserman AJ, Cotman CW. Ultrastructural analysis of beta-amyloid-induced apoptosis in cultured hippocampal neurons. *Brain Res*. 1994;661(1-2):147-56.
245. Suo Z, Fang C, Crawford F, Mullan M. Superoxide free radical and intracellular calcium mediate A beta(1-42) induced endothelial toxicity. *Brain Res*. 1997;762(1-2):144-52.
246. Saha P, Biswas SC. Amyloid-beta induced astrocytosis and astrocyte death: Implication of FoxO3a-Bim-caspase3 death signaling. *Mol Cell Neurosci*. 2015;68:203-11.
247. Stallcup WB. The NG2 proteoglycan: past insights and future prospects. *J Neurocytol*. 2002;31(6-7):423-35.
248. Ozerdem U, Monosov E, Stallcup WB. NG2 proteoglycan expression by pericytes in pathological microvasculature. *Microvasc Res*. 2002;63(1):129-34.
249. van Hinsbergh VW, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res*. 2008;78(2):203-12.
250. Jansson D, Rustenhoven J, Feng S, Hurley D, Oldfield RL, Bergin PS, et al. A role for human brain pericytes in neuroinflammation. *J Neuroinflammation*. 2014;11:104.
251. Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S. Abeta-degrading enzymes in Alzheimer's disease. *Brain Pathol*. 2008;18(2):240-52.
252. Greif DM, Eichmann A. Vascular biology: Brain vessels squeezed to death. *Nature*. 2014;508(7494):50-1.
253. Glabe CG. Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. *Neurobiol Aging*. 2006;27(4):570-5.
254. Walsh DM, Hartley DM, Condron MM, Selkoe DJ, Teplow DB. In vitro studies of amyloid beta-protein fibril assembly and toxicity provide clues to the aetiology of Flemish variant (Ala692-->Gly) Alzheimer's disease. *Biochem J*. 2001;355(Pt 3):869-77.
255. Okada T, Ikeda K, Wakabayashi M, Ogawa M, Matsuzaki K. Formation of toxic Abeta(1-40) fibrils on GM1 ganglioside-containing membranes mimicking lipid rafts: polymorphisms in Abeta(1-40) fibrils. *J Mol Biol*. 2008;382(4):1066-74.
256. Hernandez-Guillamon M, Mawhirt S, Fossati S, Blais S, Pares M, Penalba A, et al. Matrix metalloproteinase 2 (MMP-2) degrades soluble vasculotropic amyloid-beta E22Q and L34V mutants, delaying their toxicity for human brain microvascular endothelial cells. *J Biol Chem*. 2010;285(35):27144-58.
257. Qosa H, LeVine H, 3rd, Keller JN, Kaddoumi A. Mixed oligomers and monomeric amyloid-beta disrupts endothelial cells integrity and reduces monomeric amyloid-beta transport across hCMEC/D3 cell line as an in vitro blood-brain barrier model. *Biochim Biophys Acta*. 2014;1842(9):1806-15.
258. Fossati S, Cam J, Meyerson J, Mezhericher E, Romero IA, Couraud PO, et al. Differential activation of mitochondrial apoptotic pathways by vasculotropic

- amyloid-beta variants in cells composing the cerebral vessel walls. *FASEB J*. 2010;24(1):229-41.
259. Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, et al. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *J Neurosci*. 2001;21(19):7551-60.
 260. Julien O, Kampmann M, Bassik MC, Zorn JA, Venditto VJ, Shimbo K, et al. Unraveling the mechanism of cell death induced by chemical fibrils. *Nat Chem Biol*. 2014;10(11):969-76.
 261. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, et al. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature*. 1996;382(6593):685-91.
 262. Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, et al. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem*. 1994;269(13):9889-97.
 263. Susztak K, Raff AC, Schiffer M, Bottinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes*. 2006;55(1):225-33.
 264. Westfall TC, Curfman-Falvey M. Amylin-induced relaxation of the perfused mesenteric arterial bed: mediation by calcitonin gene-related peptide receptors. *J Cardiovasc Pharmacol*. 1995;26(6):932-6.
 265. Edvinsson L, Goadsby PJ, Uddman R. Amylin: localization, effects on cerebral arteries and on local cerebral blood flow in the cat. *ScientificWorldJournal*. 2001;1:168-80.
 266. Abedini A, Schmidt AM. Mechanisms of islet amyloidosis toxicity in type 2 diabetes. *FEBS Lett*. 2013;587(8):1119-27.
 267. Rawlings AM, Sharrett AR, Schneider AL, Coresh J, Albert M, Couper D, et al. Diabetes in midlife and cognitive change over 20 years: a cohort study. *Ann Intern Med*. 2014;161(11):785-93.
 268. Sanz C, Andrieu S, Sinclair A, Hanaire H, Vellas B, Group RFS. Diabetes is associated with a slower rate of cognitive decline in Alzheimer disease. *Neurology*. 2009;73(17):1359-66.
 269. Saeedi E, Gheini MR, Faiz F, Arami MA. Diabetes mellitus and cognitive impairments. *World J Diabetes*. 2016;7(17):412-22.
 270. Beltramo E, Berrone E, Tarallo S, Porta M. Different apoptotic responses of human and bovine pericytes to fluctuating glucose levels and protective role of thiamine. *Diabetes Metab Res Rev*. 2009;25(6):566-76.
 271. Janson J, Ashley RH, Harrison D, McIntyre S, Butler PC. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes*. 1999;48(3):491-8.
 272. Brender JR, Durr UH, Heyl D, Budarapu MB, Ramamoorthy A. Membrane fragmentation by an amyloidogenic fragment of human Islet Amyloid Polypeptide detected by solid-state NMR spectroscopy of membrane nanotubes. *Biochim Biophys Acta*. 2007;1768(9):2026-9.

273. Mirzabekov TA, Lin MC, Kagan BL. Pore formation by the cytotoxic islet amyloid peptide amylin. *J Biol Chem.* 1996;271(4):1988-92.
274. Brender JR, Salamekh S, Ramamoorthy A. Membrane disruption and early events in the aggregation of the diabetes related peptide IAPP from a molecular perspective. *Acc Chem Res.* 2012;45(3):454-62.
275. Rivera JF, Gurlo T, Daval M, Huang CJ, Matveyenko AV, Butler PC, et al. Human-IAPP disrupts the autophagy/lysosomal pathway in pancreatic beta-cells: protective role of p62-positive cytoplasmic inclusions. *Cell Death Differ.* 2011;18(3):415-26.
276. Zraika S, Hull RL, Udayasankar J, Aston-Mourney K, Subramanian SL, Kisilevsky R, et al. Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis. *Diabetologia.* 2009;52(4):626-35.
277. Park YJ, Lee S, Kieffer TJ, Warnock GL, Safikhani N, Speck M, et al. Deletion of Fas protects islet beta cells from cytotoxic effects of human islet amyloid polypeptide. *Diabetologia.* 2012.
278. Rapoport SI. Hypothesis: Alzheimer's disease is a phylogenetic disease. *Med Hypotheses.* 1989;29(3):147-50.
279. Finch CE, Austad SN. Commentary: is Alzheimer's disease uniquely human? *Neurobiol Aging.* 2015;36(2):553-5.
280. Ferreira PG, Munoz-Aguirre M, Reverter F, Sa Godinho CP, Sousa A, Amadoz A, et al. The effects of death and post-mortem cold ischemia on human tissue transcriptomes. *Nat Commun.* 2018;9(1):490.
281. Gerlach JC, Over P, Turner ME, Thompson RL, Foka HG, Chen WC, et al. Perivascular mesenchymal progenitors in human fetal and adult liver. *Stem Cells Dev.* 2012;21(18):3258-69.
282. Zagorski MG, Yang J, Shao H, Ma K, Zeng H, Hong A. Methodological and chemical factors affecting amyloid beta peptide amyloidogenicity. *Methods Enzymol.* 1999;309:189-204.
283. Finder VH, Vodopivec I, Nitsch RM, Glockshuber R. The recombinant amyloid-beta peptide Abeta1-42 aggregates faster and is more neurotoxic than synthetic Abeta1-42. *J Mol Biol.* 2010;396(1):9-18.
284. Brannstrom K, Ohman A, Nilsson L, Pihl M, Sandblad L, Olofsson A. The N-terminal region of amyloid beta controls the aggregation rate and fibril stability at low pH through a gain of function mechanism. *J Am Chem Soc.* 2014;136(31):10956-64.
285. Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, et al. Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes.* 2002;51(10):3107-12.
286. Lim JK, Li QX, He Z, Vingrys AJ, Wong VH, Currier N, et al. The Eye As a Biomarker for Alzheimer's Disease. *Front Neurosci.* 2016;10:536.
287. Koronyo Y, Biggs D, Barron E, Boyer DS, Pearlman JA, Au WJ, et al. Retinal amyloid pathology and proof-of-concept imaging trial in Alzheimer's disease. *JCI Insight.* 2017;2(16).

288. Logovinsky V, Satlin A, Lai R, Swanson C, Kaplow J, Osswald G, et al. Safety and tolerability of BAN2401--a clinical study in Alzheimer's disease with a protofibril selective Abeta antibody. *Alzheimers Res Ther.* 2016;8(1):14.



FACULTY OF MEDICINE

Clinical Memory Research Unit
Department of Clinical Sciences, Malmö

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2018:140
ISBN 978-91-7619-708-0
ISSN 1652-8220

