ALTERED CLOT FORMATION AND ANTICOAGULATION IN A FAMILIAL ALZHEIMER’S DISEASE MOUSE MODEL

by

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Abstract

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the leading form of dementia. Its complex etiology is traditionally attributed to an increase in the production and aggregation of amyloid-beta (Aβ). However, vascular risk factors and related disorders are also associated with the development of AD. The mechanism by which these deficiencies lead to neurodegeneration remains unclear. An altered hemostatic state has increasingly been implicated in AD pathogenesis, with the majority of research focusing on the interaction between Aβ and fibrin. It was therefore of interest to assess previously uncharacterized components of coagulation and anticoagulation in a familial AD (FAD) mouse model. Clot formation was initially analyzed using platelet rich plasma, with aged AD mice exhibiting 40% shorter clotting times compared to age-matched controls. Thrombin generation revealed differences attributable to an altered clot integrity. AD mice may form clots composed of a dense network of thin fibrin strands, resistant to fibrinolysis. Correspondingly, antithrombin (AT) activity was also reduced. These changes were subject to age, occurring specifically in aged mice only. These data suggest a change in clot formation and integrity concomitant with the development of AD. The interaction between Aβ and AT was examined in vitro, with Aβ42 and Aβ40 decreasing AT activity in a concentration dependent manner. A clinical study evaluating prothrombotic markers revealed a trend towards lower AT activity in AD patients, though not statistically significant. The changes in clot formation and anticoagulation potentiating a prothrombotic state may be specific to the FAD mouse model, but may still contribute to the elucidation of AD pathogenesis.
Preface

All of the experiments were performed by Laura Ho, in addition to the analysis of data and writing of this thesis. I designed all of the experiments with the exception of Figures 3.2 and 3.4, for which Dr. Ed Pryzdial and Dr. Christian Kastrup helped to conceptualize respectively. Figure 3.1 was generated with the help of Dr. Kaan Biron.

All animal studies were approved by the University of British Columbia’s Animal Care Committee. The animal care protocol numbers were A07-0799 and A12-0290. The study involving human patients was approved by the Clinical Research Ethics Board, protocol number H07-03022.
# Table of Contents

Abstract .......................................................................................................................... ii
Preface ............................................................................................................................ iii
Table of Contents ........................................................................................................... iv
List of Figures ................................................................................................................. vii
List of Symbols and Abbreviations ................................................................................. ix
Acknowledgements ......................................................................................................... xi

Chapter 1. Introduction .................................................................................................. 1
  1.1 Alzheimer’s disease ................................................................................................. 1
      1.1.1 Pathology of Alzheimer’s disease ................................................................. 2
      1.1.2 Genetics of familial Alzheimer’s disease and the amyloid precursor protein..... 3
      1.1.3 Amyloid precursor protein processing ......................................................... 7
      1.1.4 The amyloid cascade hypothesis ................................................................. 9
      1.1.5 Tg2576 Alzheimer’s disease mouse model .............................................. 10
  1.2 Vascular dysfunction in Alzheimer’s disease ........................................................... 11
      1.2.1 Apolipoprotein E genotype ........................................................................ 12
      1.2.2 Cerebral amyloid angiopathy .................................................................... 13
      1.2.3. Blood brain barrier dysfunction .............................................................. 14
      1.2.4. Hemostasis ............................................................................................... 15
          1.2.4.1 Primary hemostasis ........................................................................... 16
          1.2.4.2 Secondary hemostasis ...................................................................... 19
          1.2.4.3. Anticoagulation ............................................................................. 21
              1.2.4.3.1 Tissue factor pathway inhibitor ............................................... 21
              1.2.4.3.2 Antithrombin .......................................................................... 22
              1.2.4.3.3 Protein C-thrombomodulin ....................................................... 23
          1.2.4.4 Fibrinolysis ......................................................................................... 24
  1.3 Rationale .................................................................................................................. 26
  1.4 Specific aims .......................................................................................................... 28
      1.4.1 Characterization of hemostatic system activation in a mouse model of AD .... 28
      1.4.2 Assessment of coagulation system activation in patients with AD .............. 28

Chapter 2. Materials and Methods ............................................................................... 29
  2.1 Mice ......................................................................................................................... 29
  2.2 Genotyping by PCR ............................................................................................... 29
Chapter 5. Conclusions and Future Directions

5.1 Conclusions .................................................................................................................. 79

5.2 Future directions ......................................................................................................... 81

5.2.1 Characterization of the Aβ-AT interaction ................................................................. 81

Chapter 4. Discussion ...................................................................................................... 67

4.1 Accelerated clot formation and thrombin generation affecting clot integrity in Tg2576 mice .................................................................................................................. 67

4.2 Reduced antithrombin activity in Tg2576 mice ............................................................ 70

4.3 Potential interaction between Aβ and antithrombin ..................................................... 72

4.4 Thrombin generation and antithrombin activity in AD patients ................................. 76

Chapter 3. Results ........................................................................................................... 37

3.1 Characterization of hemostatic system activation in a mouse model of AD .......... 37

3.1.1 Whole blood clotting times are similar in AD and WT mice ................................. 37

3.1.2 Modified prothrombin and activated partial thromboplastin times are faster using AD plasma .................................................................................................................. 38

3.1.3 Platelet activation is similar in AD and WT mice ...................................................... 41

3.1.4 Thrombin generation is altered in aged AD mice .................................................... 43

3.1.5 Prothrombin levels in plasma are similar in AD and WT mice ............................... 50

3.1.6 Antithrombin activity is reduced in aged AD mice ................................................ 51

3.1.7 Antithrombin expression in plasma is similar between genotypes ....................... 54

3.1.8 Aβ interacts with antithrombin and lowers its activity in vitro ............................ 55

3.1.9 Amyloid peptides do not differentially affect AT activity in WT plasma .......... 58

3.2 Assessment of coagulation system activation in patients with AD ....................... 60

3.2.1 Patient overview ..................................................................................................... 60

3.2.2 Thrombin generation in AD patients results in normal maximum fluorescence and a trend towards faster activation of a thrombin substrate ................................................. 61

3.2.3 A trend towards lower antithrombin activity in AD patients relative to controls .... 65

Chapter 4. Discussion ...................................................................................................... 67

4.1 Accelerated clot formation and thrombin generation affecting clot integrity in Tg2576 mice .................................................................................................................. 67

4.2 Reduced antithrombin activity in Tg2576 mice ............................................................ 70

4.3 Potential interaction between Aβ and antithrombin ..................................................... 72

4.4 Thrombin generation and antithrombin activity in AD patients ................................. 76

Chapter 5. Conclusions and Future Directions .................................................................. 79

5.1 Conclusions .................................................................................................................. 79

5.2 Future directions ......................................................................................................... 81

5.2.1 Characterization of the Aβ-AT interaction ................................................................. 81
5.2.2 Effect of increasing AT activity in AD mice ................................................................. 82

References ......................................................................................................................................................... 84
List of Figures

Figure 1.1 Structure of the APP and location of FAD mutations which alter the production of Aβ. ................................................................. 6
Figure 1.2 The nonamyloidogenic and amyloidogenic processing of APP ................................................................. 8
Figure 1.3 The brain parenchyma is separated from intracerebral arteries and brain capillaries. 15
Figure 1.4 Layers of the endothelium and signalling molecules in response to vascular injury. 17
Figure 1.5 Platelet activation and thrombus formation ................................................................. 18
Figure 1.6 The cell based model of coagulation ................................................................. 21
Figure 1.7 Anticoagulation ................................................................. 24
Figure 3.1 Whole blood clotting times of aged AD and WT mice are similar as analyzed by in vivo tail bleed assays. ................................................................. 38
Figure 3.2 A) The reduced modified prothrombin time (PT) and B) modified activated partial thromboplastin time (aPTT) of aged AD mice are dependent on AD plasma and normal platelets ................................................................. 40
Figure 3.3 Platelet activation is similar in AD and WT mice ................................................................. 43
Figure 3.4 Aged AD mice demonstrate an initial lag in activating a fluorescent substrate for thrombin followed by a large increase in maximum fluorescence compared to WT controls ................................................................. 45
Figure 3.5 Thrombin generation in young AD and WT mice have similar activation of a fluorescent thrombin substrate and maximum fluorescence ................................................................. 46
Figure 3.6 Thrombin generation in aged AD and WT mice upon addition of unfibrillized Aβ1-42 and Aβ40-1 ................................................................. 48
Figure 3.7 Thrombin generation in aged AD and WT mice upon addition of fibrillized Aβ1-42 and Aβ40-1 ................................................................. 49
Figure 3.8 Prothrombin levels in plasma are similar in aged AD and WT mice as measured by ELISA ................................................................. 51
Figure 3.9 Antithrombin activity in AD mice is reduced in A) aged mice but not in B) young mice ......................................................................................... 53
Figure 3.10 Western blot analysis of antithrombin expression in plasma of aged and young AD and WT mice ......................................................................................... 54
Figure 3.11 Aβ peptides affect AT activity in vitro ......................................................................................... 56
Figure 3.12 Western blot analysis of AT and Aβ interaction in vitro ......................................................................................... 57
Figure 3.13 Amyloid peptides do not differentially reduce AT activity in WT plasma ......................................................................................... 59
Figure 3.14 Maximum fluorescence values achieved by the activation of a thrombin substrate are relatively normal in AD patients ......................................................................................... 63
Figure 3.15 AD and non-AD dementia patients display a trend towards shorter times to half-maximal fluorescence compared to controls.......................... 64
Figure 3.16 AD patients display a trend towards lower AT activity compared to not-cognitive impaired controls and non-AD dementia................................................................. 66
# List of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>α2AP</td>
<td>Alpha 2-antiplasmin</td>
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<td>α2M</td>
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<td>Aβ</td>
<td>Amyloid-beta</td>
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<td>ACD</td>
<td>Acid citrate dextrose</td>
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<td>ACT</td>
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<tr>
<td>AD</td>
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<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
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<td>AICD</td>
<td>Amyloid precursor protein intracellular domain</td>
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<td>APPs</td>
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<tr>
<td>aPTT</td>
<td>Activated partial thromboplastin time</td>
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<tr>
<td>AT</td>
<td>Antithrombin</td>
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</tr>
<tr>
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<td>Beta-site APP-cleaving enzyme 1</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
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</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
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<tr>
<td>CuBD</td>
<td>Copper-binding domain</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EPCR</td>
<td>Endothelial cell protein C receptor</td>
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<tr>
<td>FAD</td>
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<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GFLD</td>
<td>Growth-factor-like domain</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>HCHWA-D</td>
<td>Hereditary cerebral haemorrhage with amyloidosis Dutch type</td>
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<tr>
<td>HBD</td>
<td>Heparin-binding domain</td>
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<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
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<tr>
<td>KPI</td>
<td>Kunitz protease inhibitor</td>
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<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>LOAD</td>
<td>Late onset Alzheimer’s disease</td>
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<tr>
<td>LRP1</td>
<td>Low-density lipoprotein receptor-related protein 1</td>
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</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>NCSTN</td>
<td>Nicastrin</td>
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</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
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</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>--------------</td>
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<tr>
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<tr>
<td>PN2</td>
<td>Protease inhibitor protease-nexin 2</td>
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<td>PolyP</td>
<td>Polyphosphate</td>
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<tr>
<td>PPP</td>
<td>Platelet poor plasma</td>
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<tr>
<td>PrP</td>
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<td>Presenilin 1</td>
<td></td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
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</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
<td></td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescent unit</td>
<td></td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolytic inhibitor</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
<td></td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
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<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
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<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
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</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</tbody>
</table>
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Chapter 1. Introduction

1.1 Alzheimer’s disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder. It is the leading form of dementia, affecting approximately 30 million people worldwide [1-3]. The prevalence of dementia is estimated to double every 20 years, with a projected 81 million cases by the year 2040 [2]. Clinical symptoms of AD include a loss in memory and cognitive deficits in language, visual processing, executive function and attention [4]. The progression of dementia results in the patient becoming incapacitated prior to death, occurring on average 9 years after diagnosis [1]. The associated disability weight is higher than that of stroke, cardiovascular disease, musculoskeletal disorders and cancer, placing a heavy burden on both caregivers and health services [2]. Currently, the only predictor of AD is the occurrence of an inherited autosomal dominant mutation, affecting less than 1% of all cases in a form termed early onset familial AD (FAD). FAD is characterized by an age of onset of 30 to 50 years and is associated with an increase in amyloid-beta (Aβ) processing and aggregation [5]. In contrast, the sporadic form or late onset AD (LOAD) affects the majority of all cases and occurs after the age of 65. LOAD has a complex etiology, associated with a myriad of genetic and vascular risk factors. Recent evidence indicates that the accumulation of AD pathology commences 10 to 20 years prior to clinical manifestations, underlying the importance of understanding disease pathogenesis as well as discovering new diagnostics to define an early diagnosis of AD [3].
1.1.1 Pathology of Alzheimer’s disease

The pathology of AD was first described by Alzheimer in 1906, identifying gross cortical loss and protein aggregates in memory and cognitive-related regions of the brain [2, 3]. There are two hallmark lesions: the extracellular neuritic plaques comprised of the soluble, 4-kDa peptide amyloid-beta (Aβ) and the intracellular neurofibrillary tangles (NFTs) comprised of the phosphorylated protein tau [1]. Aβ is derived from the proteolytic cleavage of the amyloid precursor protein (APP) and polymerizes into fibrils of the β sheet conformation in the parenchyma and blood vessels of the brain [6]. It is 38-43 residues in length with Aβ40 the dominant species, accounting for nearly 90% of total Aβ secreted in the brain and cerebrospinal fluid (CSF) [3]. However, at levels of less than 10% of Aβ40, Aβ42 is thought to be the most pathogenic, its hydrophobic C-terminus making it more prone to aggregation [7].

Tau is an axonal protein which, under physiological conditions, binds to microtubules for their proper assembly and stability [4]. However, under pathological conditions, hyperphosphorylated tau dissociates from microtubules and aggregates into NFTs [3]. The density of NFTs in the cell bodies and dystrophic neurites positively correlate with disease severity compared to the number of amyloid plaques; however, due to the autosomal dominant APP and related presinilin 1 (PSEN1) or presinilin 2 (PSEN2) mutations found in FAD, Aβ has been the focus of the majority of studies relating to disease pathogenesis. In addition, NFTs are hypothesized to occur independently and after initial Aβ plaque formation, compounding disease severity through purported downstream pathways. Mutations in the tau gene result in hyperphosphorylation of tau but do not lead to AD pathology, instead resulting in frontotemporal dementia (FTD) [8, 9].
1.1.2 Genetics of familial Alzheimer’s disease and the amyloid precursor protein

The observation of similar cerebrovascular pathology in AD and Down’s syndrome led to the purification of Aβ from the amyloid plaques and the subsequent cloning of the APP [10, 11]. The APP gene is encoded by 18 exons and found on chromosome 21; triplication of the same chromosome results in Down’s syndrome [7]. Duplication of the APP gene leads to early-onset FAD with cerebral amyloid angiopathy (CAA).

APP is a type I integral membrane protein expressed primarily in the brain, but is also found in other cell types including splenocytes, leukocytes and platelets [12-14]. It is composed of a single transmembrane domain, large N-terminal extracellular domain and smaller C-terminal cytoplasmic domain (as reviewed by [15]). The ectodomain is comprised of E1 and E2 domains, containing a number of functional domains including a heparin-binding/growth-factor-like domain (HBD/GFLD), copper-binding domain (CuBD) and Kunitz protease inhibitor (KPI) domain. APP isoforms vary from 563 to 770 amino acids in length, the most common of which are the 695, 751 and 770 alternate splice variants, the former of which is primarily expressed in neurons and lacks the KPI domain present in the other two forms [12, 16]. The 751 and 770 isoforms are expressed in astrocytes, microglia and oligodendrocytes and other non-neuronal tissues such as the thymus, liver and heart. The biological function of APP has remained elusive, with studies suggesting roles in stimulating neurite outgrowth, neuronal differentiation and cell adhesion among the most compelling [17-19]. In addition, APP deficient mice reported no obvious phenotype, though there are deficits in body weight, weakened extremities and neuronal long-term potentiation [20, 21]. These abnormalities can be rescued by the soluble APP α (APPs-α) ectodomain, indicating a potential neuroprotective effect of APPs-α [22]. However,
triple knockout mice of APP and its related homologs APP-like protein-1 (APLP1) and APP-like protein-2 (APLP2)—which do not generate Aβ—exhibit postnatal lethality, implicating a yet unknown physiological role for APP [23].

Aβ is encoded by exons 16 and 17 of APP, and mutations located within or near the Aβ region are associated with FAD [24]. APP mutations are rare, occurring in only 90 families worldwide, compared to the more common PSEN mutations [25]. The identification of the first FAD mutations occurred in the early 1990s, characterizing single base pair mutations primarily at codon 717 of APP (Figure 1.1). These include the Val-Ile (London), Val-Phe (Indiana) and Val-Gly mutations (More information can be found at the Alzheimer Disease and Frontotemporal Dementia Mutation Database at http://www.molgen.ua.ac.be/ADMutations/) [26, 27]. Located near the C-terminus of the Aβ region, they are associated with altered γ-secretase processing and result in an increased Aβ42 to Aβ40 ratio [28]. However, overall expression of Aβ remains largely unaffected [3]. In contrast, the Swedish mutation (K670N/M671L) is located near the N-terminus of Aβ. It affects β-secretase cleavage and therefore increases not only Aβ42 expression but also all other Aβ species as well. The Swedish mutation is also associated with cerebral amyloid angiopathy (CAA). Currently, 33 APP mutations have been identified, the majority of which are located within the transmembrane domain near the C-terminus.

Following the discovery of the first APP mutations, PSEN1 and PSEN2 were identified to also contain causal AD mutations. Highly homologous, PSEN1 and PSEN2 are located on chromosome 14 and 1 respectively, encoding membrane proteins important for γ-secretase processing of APP [29, 30]. Their mutations result in increased Aβ42 production, altering the Aβ42 to Aβ40 ratio both in vitro and in vivo [31]. PSEN1 mutations account for the largest
proportion of FAD cases. They are associated with a very early age of onset, with some individuals affected in their twenties [32]. In contrast, PSEN2 mutations account for the smallest number of FAD cases with a delayed age of onset compared to PSEN1 and APP mutations [5].
Figure 1.1 Structure of the APP and location of FAD mutations which alter the production of Aβ.

A) Structure of the transmembrane APP from which Aβ is derived. B) FAD mutations occurring within or adjacent to Aβ result in an increased production and aggregatability of Aβ. The Flemish and Dutch mutations decrease α-secretase activity, shifting proteolytic processing from the nonamyloidogenic pathway to the amyloidogenic pathway. In contrast, the Swedish mutation increases β-secretase activity resulting in an increase of all Aβ species. The PSEN, British and Indiana mutations increase γ-secretase activity, altering the production of Aβ40 and Aβ42 specifically. Reprinted with permission from the Journal of Clinical Investigation, 2005 [25].
1.1.3 Amyloid precursor protein processing

APP is processed through two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway. Both pathways are initiated by a cleavage within the luminal domain (Figure 1.2). In the non-amyloidogenic pathway, α-secretase cleaves APP between residues 16 and 17 of the Aβ sequence, precluding the generation of Aβ while liberating a large soluble APP (APPs-α) extracellular domain and a membrane-associated C-terminal fragment of 83 residues (C83) [33]. Subsequent γ-secretase cleavage of C83 releases the APP intracellular domain (AICD) and hydrophobic, 3 kDa p3 fragment. α-secretase activity is controlled by the ADAM (a disintegrin and metalloprotease) family of proteases. Increasing α-secretase activity may decrease amyloidogenic processing of APP, though the effects of upregulating non-amyloidogenic metabolic products remain unclear. The non-amyloidogenic pathway accounts for 90% of APP processing, with the remaining 10% processed via the amyloidogenic pathway [34].

In the amyloidogenic pathway, β-secretase cleaves APP near the N-terminus of the Aβ sequence, releasing a APPs-β ectodomain and carboxy terminal fragment C99. Subsequent γ-secretase cleavage of C99 releases the AICD and Aβ peptide [7, 33]. β-secretase activity is regulated by β-site APP-cleaving enzyme 1 (BACE1), a type 1 transmembrane aspartic protease [35]. BACE1 is a major target of pharmacologic inhibition due to its ability to generate toxic Aβ species. Its specificity is also appealing, as APP is one of only two known BACE1 substrates [25]. Mouse models have supported this theory, with bigenic BACE1-null, APP-expressing mice exhibiting decreased cerebral Aβ40 and Aβ42 levels and ameliorating cognitive deficits [36]. However, clinical trials have been less encouraging, with toxicity and central nervous system (CNS) exclusion concerns limiting their success [25].
Figure 1.2 The nonamyloidogenic and amyloidogenic processing of APP.

APP can be processed via A) the nonamyloidogenic pathway or B) the amyloidogenic pathway. A combination of $\alpha$ and $\gamma$-secretase activity results in the generation of p3, while a combination of $\beta$ and $\gamma$-secretase activity results in the generation of A$\beta$. C) $\gamma$-secretase can cleave APPs-$\alpha$ or APPs-$\beta$ at multiple sites, forming A$\beta$ species of varying lengths of which the 40 and 42 residue are the most common. Reprinted with permission from Macmillan Publishers Ltd (Nature Reviews: Neurology), copyright (2010) [33].
γ-secretases have also received attention in drug therapy. The γ-secretase complex is composed of a catalytic core (PSEN1 and PSEN2) and three accessory proteins [nicastrin (NCSTN), anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN2)] [33]. Modulating γ-secretase proteolysis has proven challenging, as it can cleave at multiple sites within APP [25]. In addition, it contains a diverse range of substrates including Notch and therefore its alteration can result in adverse side effects. A recent Phase III clinical trial in which γ-secretase activity was the therapeutic target proved unsuccessful due its non-specific substrate proteolysis [37].

1.1.4 The amyloid cascade hypothesis

The prevailing view of disease pathogenesis is based on the molecular defects of FAD, the clinical manifestation of which mirrors LOAD. The amyloid cascade hypothesis proposes that the accumulation of Aβ initiates downstream events, including tau tangles, synaptic dysfunction and neuronal loss leading to cognitive decline [38]. It relates to the dysregulation in the production and clearance of Aβ. The fully penetrant mutations in APP, PSEN1 and PSEN2 all support this theory, whereby the production of the aggregation-prone Aβ42 is increased. In addition, recent studies indicate that amyloid deposition commences 10 to 20 years prior to the development of clinical symptoms, well before the appearance of NFTs [3].

Formulated over 20 years ago, major inconsistencies in the amyloid cascade hypothesis have emerged with advancing research. Firstly, plaque burden does not correlate well with disease severity; tau tangles as well as soluble Aβ are much better markers of AD progression. The distribution of tau pathology is well-characterized, corresponding to six stages of disease
progression and used as a diagnostic tool [39]. Soluble Aβ is also used in the clinic, where its concentration in the cerebrospinal fluid (CSF) is an established biomarker. The decreasing ratio of Aβ42 to Aβ40 in the CSF is thought to be indicative of the sequestration and incorporation of Aβ42 into amyloid plaques. Clinical trials have tested the amyloid cascade hypothesis with largely inconclusive results. An active Aβ42 immunization study in 2008 demonstrated a significant reduction in plaque load in eight patients who had come to autopsy—however, these patients still developed tau pathology in the primary cortex not initially present at the start of the study (Braak stage IV-V) [9, 40]. In addition, clinical progression of AD remained unaffected. Another concern of the hypothesis involves the phenotype of transgenic mice expressing mutant APP, PSEN1 or PSEN2, which develop amyloid plaques but fail to recapitulate NFTs or neuronal loss [41]. Despite these developments, the amyloid cascade hypothesis remains critically important, providing the basis for pharmacologic intervention.

1.1.5 Tg2576 Alzheimer’s disease mouse model

AD animal models are primarily based in origin on the genetics of FAD. The Tg2576 mouse model was used for the experiments described below. Under the control of the hamster prion protein (PrP) promoter, it overexpresses the human 695 amino acid isoform of APP with the K670N/M671L Swedish mutation, in which two amino acids immediately adjacent to the N-terminus of the Aβ region are mutated [16, 42]. Originally found in a Swedish FAD family, the mutation causes an accelerated age of onset of 55 years [24], and in vitro is associated with a 6 to 8 fold increase in Aβ expression compared to the non-mutated form of APP [42]. The Swedish mutation increases β-secretase activity and, unlike mutations located in the Aβ domain, does not
It is possible to discriminate between different Aβ species.

In the Tg2576 mouse model, Aβ deposition occurs at 9 months of age concomitant with the development of CAA [16, 43]. The pathology also corresponds to the presentation of cognitive and behavioural deficits. BBB dysfunction is detected as early as 4 months of age, preceding Aβ deposition in the brain and cognitive decline [44]. Tg2576 mice exhibit high levels of plasma Aβ (4.5 nM) compared to other Swedish mutation models (pM range) [43]. This may be due to the specificity of their promoters, with the hamster PrP promoter demonstrating increased peripheral activity compared to the neuronal-specific activity of the murine Thy1 promoter in the APP23 and TgArcSwe models. In addition, CSF levels of Aβ42 decrease with age, mirroring the biochemical progression of AD. Pathogenesis is similarly accelerated in female mice. Though the Tg2576 model exhibits Aβ neuropathology and accompanying dementia, it does not present with NFTs and neuronal loss [1]. Currently, there is no mouse model that recapitulates the pathology of AD in its entirety.

1.2 Vascular dysfunction in Alzheimer’s disease

Vascular dysfunction has recently been implicated in disease pathogenesis. Risk factors for AD include hypertension, hypotension, smoking, hypercholesterolemia and type II diabetes mellitus [45]. In addition, the apolipoprotein E (ApoE) ε4 allele is a strong genetic risk factor and supports the relationship between vascular disease and the development of AD. These risk factors are also associated with cerebral amyloid angiopathy (CAA), a related amyloid deposition condition. Furthermore, a compromised blood brain barrier (BBB) may lead to the
dysregulation of Aβ and contribute to the extravasation of plasma proteins into the brain parenchyma, with studies suggesting an altered hemostatic system both in vitro and in vivo [44, 46, 47]. These vascular deficiencies may contribute to both the development and progression of AD, underlying the importance of understanding the relationship between vascular dysfunction and neurodegeneration.

1.2.1 Apolipoprotein E genotype

ApoE encodes a plasma cholesterol transport protein and the allelic segregation of its three variants (ε2, ε3 and ε4) greatly impacts the risk of developing AD [48]. ApoE ε2 confers a protective role against amyloid deposition, while ApoE ε4 is associated with increased plaque burden and NFTs [49]. The presence of one copy of the ε4 allele increases the risk of developing AD three-fold, while two copies increases the risk 9- to 10-fold [25]. ApoE is also implicated in FAD, accelerating the age of onset of mutant PSEN carriers [50].

ApoE is primarily expressed by astrocytes in the brain, but can also be expressed by other non-neuronal cells including microglia [51]. It is involved in maintaining synaptic function in response to injury, and its receptor-mediated endocytosis is regulated by low-density lipoprotein receptor-related protein 1 (LRP1). ApoE ε4 is hypothesized to alter Aβ binding, affecting its metabolism through decreased clearance mechanisms (reviewed by [52]). It is also implicated in β sheet formation, Aβ aggregation and tau phosphorylation. ApoE genotype is a risk factor for vascular diseases including atherosclerosis and stroke [51]. It is also strongly correlated with the development of CAA, with ε4 carriers displaying greater cerebrovascular Aβ deposition compared to non-ε4 carriers [53, 54].
1.2.2 Cerebral amyloid angiopathy

Cerebral amyloid angiopathy (CAA) is characterized by Aβ deposition in the leptomeningeal and parenchymal arteries, arterioles and capillaries, resulting in the thickening of their vessel walls, degeneration of smooth muscle cells and formation of micro-aneurysms [45, 55]. It can also lead to lobar hemorrhage and hemorrhagic stroke [56]. CAA affects 70 to 97.6% of AD patients, but can also occur independently of AD [55]. The occurrence of age-related CAA increases to almost 100% in the elderly over the age of 80. Three hypotheses have been proposed to account for the deposition of Aβ in CAA: 1) the systemic production of Aβ from the circulation, 2) the generation of Aβ from smooth muscle cells within the vessel walls and pericytes and 3) the production of Aβ from neurons with impaired perivascular drainage across the BBB.

Though CAA is primarily sporadic in origin, mutations in the APP gene can lead to familial CAA. For example, hereditary cerebral haemorrhage with amyloidosis Dutch type (HCHWA-D) is an autosomal dominant form of CAA, in which a mutation located in the Aβ region results in fatal cerebral haemorrhages between the ages of 45 to 55 [6, 57, 58]. Patients present with diffuse amyloid plaques but without NFTs and accompanying dementia typical of AD. ApoE genotype also plays a role in the severity of CAA. Though its frequency is low in CAA-related cases, the ApoE ε2 allele is associated with increased CAA-related intracerebral hemorrhage due to vessel wall damage [53]. As a result, antiplatelet and anticoagulant therapies pose a risk for ε2 carriers compared to non ε2 carriers [53, 59].
1.2.3. Blood brain barrier dysfunction

The BBB is composed of endothelial cells which form a membrane surrounding cerebral capillaries, compartmentalizing the brain from the peripheral blood (as reviewed by [60]). Its integrity is mediated by both neuronal and non-neuronal cells including pericytes and astrocytes (Figure 1.3). The BBB limits the entry of plasma proteins, red blood cells (RBCs) and leukocytes into the brain while facilitating the selective transport of metabolites and nutrients required for neuronal processes. A compromised BBB results in the aberrant transport of molecules into and out of the brain parenchyma, concomitant with inflammation, angiogenesis and reduced neuronal survival [45].

BBB dysfunction is associated with age-related changes in vascular permeability in humans in addition to ApoE genotype and CAA in rodent models [60-62]. It may also contribute to neuronal degeneration in AD through its dysregulation of Aβ. Aβ influx is mediated by the receptor for advanced glycation end products (RAGE). Its expression is increased in neurons, vascular cells and glia in AD, resulting in an accumulation of Aβ in the brain. In contrast, LRP1 regulates Aβ efflux and its expression is decreased in AD. Studies both in human and animal models of AD demonstrate a reduced ability to clear Aβ from the BBB. In addition, mice expressing low LRP1-clearing Aβ develop accelerated amyloid deposition compared to controls [63].
Figure 1.3 The brain parenchyma is separated from intracerebral arteries and brain capillaries.

The brain parenchyma is separated from the penetrating intracerebral arteries and brain capillaries to regulate the selective transport of cells and molecules into and out of the brain. Intracerebral arteries are separated by the glia limitans, composed of a single layer of fibroblast-like cells. The support of the glia limitans does not extend to the level of the capillaries, which are instead separated from the parenchyma by a layer of vascular endothelial cells (the BBB). Pericytes surround the endothelial cells, and astrocytes encase the endothelial cells and pericytes. Reprinted with permission from Macmillan Publishers Ltd (Nature Reviews: Neuroscience), copyright (2011) [60].

1.2.4. Hemostasis

Hemostasis is a physiological process that maintains blood in a fluid state, and acts to limit blood loss upon injury to the blood vessel endothelium. Maintaining the systemic circulatory system requires a delicate balance between procoagulation and anticoagulation, with multiple feedback loops and pathways ensuring its success. Its dysregulation results in bleeding complications or thrombotic disorders.
Hemostasis can be divided into three phases. In primary hemostasis, platelets adhere to the site of injury to form a hemostatic plug. Secondary hemostasis involves the activation of the coagulation cascades, culminating in the generation of a fibrin clot. Fibrinolysis, the final stage, describes the dissolution of the clot.

1.2.4.1 Primary hemostasis

Platelet thrombus formation is central in mediating hemostatic responses upon injury to the blood vessel endothelium. Vascular injury exposes the adhesive proteins von Willebrand factor (VWF) and collagen, resulting in the activation of receptors present on the platelet surface. Stable adhesion occurs through the initial tethering and rolling of platelets to the extracellular matrix (ECM) [64]. At high shear rates or pathological conditions, such as those found in small arteries or atherosclerotic vessels, platelets must slow down by rolling over collagen or ECM-bound VWF. This prolongs the contact between platelets and components of the ECM, facilitating platelet-ECM interactions. In the high shear arterial system, adhesion is almost exclusively regulated by GPIbα, part of the GPIb-IX-V receptor complex. In the low shear arterial system, present in veins and large arteries or after the initial reduction in platelet velocity, adhesion is mediated by the receptor GPVI or the integrin α2β1 through their interaction with collagen [65]. Platelet adhesion leads to the activation of tyrosine kinase or G-protein coupled receptor (GPCR) signalling cascades, granule release, recruitment of additional platelets and platelet aggregation [64]. Platelet-platelet interaction occurs through the inside-out signalling of the integrin αIIbβIII. The symmetrical nature of fibrinogen facilitates the cross-linking of
αIIbβIII molecules on adjacent platelets, forming platelet aggregates. This results in the generation of a fibrin-rich hemostatic plug.

Figure 1.4 Layers of the endothelium and signalling molecules in response to vascular injury.

Under physiological conditions, signaling molecules initiating platelet activation or coagulation are not exposed to the circulatory system. However, upon injury to the endothelium, VWF and collagen become exposed from the subendothelial intima to activate receptors on the platelet surface. TF, located in the adventitial layer of the vessel wall, is also exposed, triggering coagulation. Reprinted with permission from Topics in Companion Animal Medicine, 2012 [66].
Vascular injury exposes VWF and collagen, resulting in the activation of receptors (GPIb-V-IX and GPVI) present on the platelet surface. Stable adhesion occurs through the initial tethering and rolling of platelets to the ECM. Platelet activation is followed by granule secretion and aggregation. The release of autacoids or soluble secondary mediators such as ADP and thromboxane A2 can also stimulate platelet activation. In addition, platelets provide a procoagulant surface for the generation of thrombin. This results in the formation of a fibrin-stabilized thrombus. Platelets also interact with thrombin through GPCRs in a positive feedback reaction. The heat map indicates the developing calcium concentration, with green representing low Ca$^{2+}$ concentration and red representing high Ca$^{2+}$ concentration. Reprinted with permission from the American Physiological Society, copyright (2013) [67].
1.2.4.2 Secondary hemostasis

Secondary hemostasis can be divided into three phases of coagulation: initiation, amplification and propagation (as reviewed by [66]). It involves two coagulation pathways—the extrinsic (tissue factor) pathway and the intrinsic (contact activation) pathway. These series of tightly regulated enzymatic reactions converge in the common pathway, culminating in the generation of thrombin and the formation of a fibrin clot.

The initiation phase of coagulation occurs when tissue factor (TF), an extravascular integral membrane protein, is exposed to blood upon injury to the endothelium [66]. In the extrinsic pathway, TF forms a complex with plasma factor (F) VII/VIIa, leading to the activation of factors IX and X. Factor Xa converts prothrombin to thrombin in trace amounts.

In the amplification phase, the accumulation of thrombin becomes sufficient to activate cofactors V and VIII. Thrombin also activates factor XI. Platelets at the site of injury provide a phospholipid membrane surface on which the factors can assemble [65]. The procoagulant surface also exposes anionic phospholipid, necessary for the binding of cofactors and serine proteases.

In the propagation phase of coagulation, the tenase complex, composed of factors VIIIa, IXa and X, amplifies the production of factor Xa [66]. The prothrombinase complex, composed of factors II (prothrombin), Va and Xa, generates a burst of thrombin. Thrombin converts fibrinogen to fibrin which is subsequently cross-linked by factor XIIIa, resulting in the formation of a stable clot.

Fibrin formation occurs primarily through the extrinsic pathway in vivo, with the physiological relevance of contact factors currently unclear. The intrinsic pathway is
hypothesized to contribute to pathological thrombus formation through the amplifying tenase complex [68]. It is initiated when blood comes into contact with a negatively charged surface, leading to the sequential activation of factors XII, XI and IX. This theory is supported by the relatively normal bleeding tendencies of factor XII deficient patients. However, animal studies suggest factor XII deficiency protects against arterial thrombus formation and ischemic brain injury [69].

Polyphosphate (polyP) may be the physiological activator of factor XII [69]. An inorganic, negatively charged polymer of 60 to 100 residues, polyP is released from the dense granules of activated platelets and initiates platelet-induced fibrin formation and vascular leakage. In addition to activating factor XII, it also accelerates other procoagulant mechanisms by enhancing factor Xa or thrombin-mediated factor Va activation [70]. As a result, tissue factor pathway inhibitor (TFPI) anticoagulant activity is inhibited while thrombin activatable fibrinolytic inhibitor (TAFI) antifibrinolytic activity is increased.
There are three phases in the cell based model of coagulation:

A) In the initiation phase, vessel injury exposes TF which initiates coagulation. TF forms a complex with factor VII/VIIa, leading to the activation of factors IX and X. Factor Xa slowly activates factor V and converts a small amount of prothrombin to thrombin.

B) In the amplification phase, the accumulation of thrombin activates cofactors V and VIII, enhancing the catalytic activity of factors IXa and Xa. Platelets provide a phospholipid surface on which the factors can assemble.

C) In the propagation phase, the tenase complex (factors VIIIa, IXa and X) activates factor X, which binds to factor Va and prothrombin to form the prothrombinase complex. This generates a burst of thrombin, resulting in fibrin deposition. Reprinted with permission from Macmillan Publishers Ltd (Nature Reviews: Cardiology), copyright (2012) [71].

1.2.4.3. Anticoagulation

1.2.4.3.1 Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) is one of three main natural anticoagulants present in the circulatory system and is the major inhibitor of TF. A Kunitz-type protease inhibitor, it
mediates TF-FVIIa activity via two stages of inhibition—the reversible inhibition of factor Xa followed by the binding of the TFPI-FXa complex to the TF-FVIIa complex (as reviewed by [72]). Its purpose is to limit the initiation phase of coagulation, when only trace amounts of thrombin are formed, preventing unnecessary factor IX and X-mediated thrombin generation [66].

### 1.2.4.3.2 Antithrombin

Antithrombin (AT), also termed AT III, is a serine protease inhibitor and the major physiological inhibitor of thrombin (as reviewed by [73]). In addition, it also inhibits factors IXa, Xa, XIa and XIIa. Its mechanism of action is enhanced 1000-fold by its cofactor heparin, underlying the clinical use of heparin as an anticoagulant. Endogenous heparan sulfate, closely related to heparin, is the physiological activator of AT in vivo [74]. Heparans are glycosaminoglycans expressed on the surface of endothelial cells [75]. Only a fraction of heparans contain acidic, negatively charged repeating pentasaccharide sequences which interact with the basic, positively charged heparin-binding domain of AT for its allosteric activation [73]. Heparin binding results in a conformational change in AT, increasing the proximity of its active site to that of the proteinase. For factors IXa and Xa, binding of the heparin pentasaccharide sequence is sufficient to enhance their reactivity with AT. However, thrombin inhibition is specific in that it also requires full-length glycosaminoglycans of at least 18 saccharides or longer for the formation of a ternary bridging complex between thrombin, AT and heparin [74, 76]. The extended polysaccharide chain provides an additional negatively charged exosite adjacent to bound AT which promotes the recruitment of thrombin. In contrast, the bridging
effect of longer glycosaminoglycans with factors IXa and Xa serves to augment AT interactions. After the release of heparin, AT forms a 1:1 covalent complex with thrombin (or with other serine proteases), irreversibly inhibiting the enzyme.

1.2.4.3.3 Protein C-thrombomodulin

The amplifying prothrombinase and tenase complexes are inhibited via the protein C anticoagulant pathway (as reviewed by [77]). Protein C (PC) is a vitamin K-dependent glycoprotein with high homology to the procoagulant serine proteases including factors VII, IX and X [67]. Thrombin forms a complex with the cofactor thrombomodulin (TM), which subsequently activates PC to activated protein C (APC). This interaction is enhanced 20-fold if PC is bound to the endothelial cell protein C receptor (EPCR) [77]. After dissociating from the EPCR, APC binds to protein S to inactivate factors Va and VIIIa, inhibiting prothrombinase and tenase formation. The inactive form of factor V also serves as a cofactor for APC-mediated proteolysis of factor VIIIa. The major protease inhibitors of APC include protein C inhibitor, α1-proteinase inhibitor and α2-macroglobulin.
Anticoagulation occurs through 3 main mechanisms. TFPI binds to FXa and the TF-FVIIa complex, limiting coagulation initiation. Protein S acts as a cofactor for TFPI, increasing its affinity for FXa. Anticoagulation can also occur through AT, a serine protease inhibitor which inhibits factors IIa (thrombin), Va, VIIIa, IXa and Xa. The procoagulant function of thrombin is altered when bound to TM, which activates PC to APC. This interaction is greatly enhanced when PC is bound to EPCR. APC, in complex with its cofactor PS, inactivates the amplifying prothrombinase and tenase complexes. Reprinted with permission from the American Physiological Society, copyright (2013) [67].

1.2.4.4 Fibrinolysis

Fibrinolysis encompasses the dissolution of a clot (as reviewed by [78]). It is primarily mediated by the serine protease plasmin, which circulates in plasma in its zymogen form plasminogen. Fibrin acts as a cofactor for the activation of plasminogen as well as a substrate for
plasmin [79]. Initially, trace amounts of plasmin are generated from plasminogen localized to the fibrin clot [78]. Its conversion is mediated by tissue-type plasminogen activator (tPA), but can also occur via other plasminogen activators including urokinase-type plasminogen activator (uPA). Plasmin proteolysis of fibrin results in the exposure of C-terminal lysines and the formation of a complex composed of plasminogen, tPA and primed fibrin. The ternary complex accelerates the activation of plasmin, which in turn exposes additional binding sites for plasminogen and tPA. Plasmin then degrades polymerized fibrin into fibrin degradation products. This process is auto-regulated, with decreasing fibrin limiting its role as a cofactor for plasminogen. It also serves to inhibit the fibrinolytic system in the absence of a fibrin clot.

Inhibitors of fibrinolysis include α2-antiplasmin (α2AP), α2-macroglobulin (α2M), plasminogen activator inhibitor type 1 (PAI-1) and thrombin activatable fibrinolytic inhibitor (TAFI). α2AP is a serine protease inhibitor and the primary physiological inhibitor of plasmin. It forms a complex with plasmin through its C-terminal lysine, preventing the lysine binding site of plasmin from interacting with fibrin. In addition, α2AP is cross-linked to fibrin by factor XIII, resulting in a clot resistant to lysis. α2M is a glycoprotein with a wide range of substrates. It inhibits plasmin through the formation of a non-covalent complex [80]. α2M also exerts an anticoagulant function through its interaction with the exosite of thrombin, inhibiting subsequent substrate activation [81]. PAI-1 is a serine protease inhibitor, forming an irreversible 1:1 complex with plasminogen activators tPA and uPA. It acts to prevent premature lysis of a developing clot. TAFI is a procarboxypeptidase B which is activated by plasmin and thrombin-TM. It inhibits fibrinolysis through the removal of C-terminal lysines on fibrin, preventing the binding of plasminogen to fibrin.
1.3 Rationale

Vascular dysfunction as a result of its related risk factors and disorders may contribute to disease pathogenesis. In addition, an altered hemostatic system is increasingly implicated in AD. Though some data suggest an anticoagulant function of APP, the majority of research supports an association of procoagulant mechanisms with AD.

Platelets release APP upon α-granule secretion, and Aβ fibrils can stimulate platelet adhesion and aggregation [82]. In addition, APP isoforms expressing a KPI domain are analogous to the protease inhibitor protease-nexin 2 (PN2) [83]. The KPI domain is hypothesized to inhibit factors IXa, Xa and XIa. Mice with modest overexpression of a KPI-containing APP isoform in platelets exhibit delayed cerebral thrombosis, with larger hematoma volumes and slower times to clot formation compared to controls. However, the anticoagulant function of APP is limited to its non-neuronal expression of KPI-containing isoforms.

Thrombin is also associated with AD pathology, present in neuritic plaques and NFTs of AD patients [84]. In addition, prothrombin accumulation in the microvasculature of the prefrontal cortex correlates with Braak stage progression and ApoE genotype [85]. Prothrombin and thrombin are also expressed at the mRNA level in neurons and glial cells including astrocytes [86]. In vitro, thrombin is able to cleave tau at multiple sites resulting in its degradation [87]. However, the phosphorylation of tau inhibits its proteolysis, potentially resulting in its aggregation and the formation of tau tangles.

A connection between fibrin, CAA and AD has also been established. Fibrin co-localizes with CAA positive vessels in a mouse model of AD, and pharmacologic depletion of fibrin...
lessens CAA pathology and improves cognitive impairment [47]. Intravital imaging also revealed abnormal clot formation and dissolution in the same model. In vitro, Aβ42 alters the structure of a fibrin clot, resulting in aggregated fibrils resistant to lysis. It also induces fibrinogen oligomerization through interactions with the C terminus of the β chain of fibrinogen [88]. In accordance, the fibrinolytic system is also altered in AD. tPA may contribute to the degradation of Aβ in vivo and its activity is diminished in AD [89, 90]. Plasmin is also downregulated, while its inhibitor PAI-1 is correspondingly upregulated. These changes in expression occur in the hippocampus and amygdala, consistent with regions affected in AD.

In combination, these data suggest an altered hemostatic system with abnormal thrombin generation and fibrin formation leading to clot structures resistant to degradation. However, the mechanism by which Aβ may induce cerebrovascular abnormalities leading to neurodegeneration remains unclear. We hypothesize that a compromised BBB results in the extravasation of plasma factors and their inhibitors into the brain parenchyma, potentiating a prothrombotic state. Alterations in procoagulant and anticoagulant mechanisms may elucidate pathogenesis in AD.

The objective of this study was to characterize clot formation in AD. The Tg2576 mouse model of AD was used for the majority of the experiments described. Although the Tg2576 mouse model reflects the rare familial form of AD in origin, its biochemical and pathological changes also mirror that of the sporadic form of AD. The majority of previous studies characterizing clot formation and dissolution have been analyzed primarily in vitro, using synthetic peptides as a source for endogenously-derived Aβ in plasma. In contrast, the experiments described below use plasma from Tg2576 mice and demonstrate the effect of using
different sources of Aβ. Thrombin and its physiological inhibitor antithrombin (AT) proved to be useful biomarkers of disease progression in the Tg2576 mouse model, and were therefore used in a blinded study of AD, not cognitive impaired and non-AD dementia-diagnosed patients.

1.4 Specific aims

1.4.1 Characterization of hemostatic system activation in a mouse model of AD

Hemostatic system activation in the Tg2576 mouse model of AD was analyzed using experiments based on clinical parameters of hemostasis including modified clotting times, thrombin generation and AT activity. Accelerated clot formation was indicative of altered clot integrity and anticoagulation, posing a potential thrombotic risk to AD mice.

1.4.2 Assessment of coagulation system activation in patients with AD

Coagulation system activation was assessed using thrombin generation and AT activity in a cohort of 83 AD, not cognitive impaired and non-AD dementia patients. Data were specifically analyzed for patients in their sixties to minimize the variation in age distribution between groups. Results indicated a trend towards differences in thrombin generation and lower AT activity in AD patients compared to not cognitive impaired controls.
Chapter 2. Materials and Methods

2.1 Mice

Tg2576 mice were a kind gift from Dr. Karen Hsiao, housed at the University of British Columbia Small Animal Facility at South Campus and the Animal Resource Unit. Mice were maintained on a C57Bl6/SJL background by mating hemizygous transgene positive male offspring to C57Bl6/SJL F1 female hybrids (The Jackson Laboratory). Transgene negative littermates were used as wild-type (WT) controls. All mice were fed standard lab chow and water ad libitum and kept under a 12 hour light/dark cycle. Experimental procedures were approved by the University of British Columbia Animal Care Committee in accordance with the regulations of the Canadian Council on Animal Care. Mice 18 months or older were considered aged, while young mice were between 3 and 5 months of age.

2.2 Genotyping by PCR

Mice were genotyped by PCR using ear notches. Ear notches were incubated with 20 μl of digestion buffer (50 mM Tris pH 8.0, 2 mM NaCl, 10 mM EDTA, 1 % SDS, 1 mg/ml Proteinase K) for 2 hours at 55°C with periodic vortexing. Afterwards, the sample was diluted with 500 μl of Milli-Q H2O and the enzyme inactivated by incubating the reaction for 10 minutes at 95°C. The genomic DNA was used to detect the presence of the APP transgene as well as an internal control. Primers previously described [91], corresponding to the 3’ region of the human APP gene (5’-CTGACCACACTCGACCAGGGTTCTGGGT-3’) and to the 3’ untranslated region of the
hamster PrP promoter (5'-GTGGATAACCCCTCCCCCAGCCTAGACCA-3’), were used to amplify the PrP-APP fusion gene (amplicon of approximately 500 base pairs). Primers (5’-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3’) and (5’-GTGGATAACCCCTCCCCCAGCCTAGACCA-3’) amplified a region of the murine PrP as a positive control (amplicon of approximately 800 base pairs).

2.3 Whole blood clotting times

Aged AD and WT mice were terminally anaesthetized with avertin (0.02 mL/1g) and placed in a modified 50 ml Falcon tube as a restrainer. The tails of AD mice and WT littermates were transected at a diameter of 1 mm from the tip with a scalpel and immediately immersed in saline (37°C). The time until cessation of bleeding was recorded until a maximum time of 20 minutes.

2.4 Modified activated partial thromboplastin time and prothrombin time

Blood from aged AD and WT mice was collected via saphenous venipuncture using 25 G x 5/8 inch needles into EDTA-coated collection tubes (BD Biosciences) for a maximum blood loss equal to 10% of total blood volume (7 mL/kg). Blood was centrifuged for 15 minutes at 150 x g to isolate platelet rich plasma (PRP). PRP was pooled for AD or WT mice as volumes from each mouse were too small to evaluate individually. Pooled PRPs were then acidified with half (PRP) volumes of acid citrate dextrose [(11.5 mM citrate acid monohydrate, 88.5 mM trisodium citrate dehydrate and 111 mM dextrose, pH 6.0)] and divided into two aliquots. One aliquot of each
genotype was set aside for experimental use, while the other was centrifuged for 10 minutes at 750 x g to separate platelets from platelet poor plasma (PPP). Platelets of either AD or WT origin were then resuspended in the PPP of the opposite genotype for non-physiological PRP.

Samples were analyzed in duplicate using a START4 Coagulation Analyzer (Diagnostica Stago). Prothrombin time (PT) was measured by incubating 15 µl of either physiological PRP or platelets resuspended in PPP of the opposite genotype with 65 µl HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 1 minute at 37°C. 40 µl of Innovin (Dade Behring), a source of recombinant human tissue factor, synthetic phospholipids and calcium, was added and the time to clot formation recorded. Activated partial thromboplastin time (aPTT) was measured by incubating 15 µl of sample with 45 µl HEPES buffer and 30 µl of APTT reagent (a silica activator) (Stago) for 3 minutes at 37°C. Afterwards, 30 µl of 25 mM CaCl₂ was added and the time to clot formation recorded.

2.5 Platelet activation by flow cytometry

Blood citrated 9:1 (v/v) with ACD was collected via cardiac puncture (25 G x 5/8 inch needle, 1mL syringe) of aged AD and WT mice into 1.5 mL Eppendorf tubes. Platelets were isolated by two consecutive centrifugations of 15 minutes at 150 x g and 10 minutes at 750 x g, acidifying the sample with half (PRP) volumes of ACD between each step. After isolation, platelets were washed twice with 200 µl CGSA buffer (10 mM trisodium citrate, 30 mM dextrose, 120 mM NaCl, pH 6.5) in the presence of apyrase (1 U/mL, Sigma) to minimized platelet activation and resuspended in HEPES buffer at physiological concentrations. Washed
platelet resuspensions were recalcified (1.8 mM CaCl₂), incubated for 10 minutes at 37°C (600 rpm) and stimulated with thrombin (1 U/mL) (Sigma). After a second incubation of 10 minutes at 37°C (600 rpm), samples were stained with antibodies against FITC-labeled mouse P-selectin (CD62P, BD Biosciences) and PE-labeled mouse GPIIbIIIa (clone JON/A, Emfret Analytics) at room temperature for 10 minutes. The reaction was stopped with the addition of 450 μl of 1 % paraformaldehyde (v/v) in HEPES. Platelets from individual mice were analyzed separately (no pooling of samples). Data were acquired using LSRII/FACSDiVa software (BD Biosciences) and analyzed with the FlowJo program (Treestar Inc.).

2.6 Thrombin generation

Blood citrated 9:1 (v/v) from aged and young AD and WT mice was obtained via cardiac puncture and plasma isolated by centrifugation for 10 minutes at 10 000 g. 60 μl of pooled plasma samples were recalcified with the addition of 26 μl of 40 mM CaCl₂, 90 mM NaCl and 0.4 mM of t-butyloxycarbonyl-β-benzyl-L-aspartyl-L-prolyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA, Peptides International), a fluorescent substrate for thrombin (excitation wavelength of 390 nm and emission wavelength of 460 nm). Plasma was activated with a source of recombinant tissue factor and synthetic phospholipids (diluted Innovin) and the reaction was allowed to proceed for a maximum of 60 minutes at 37°C. For reactions involving the addition of unfibrillized amyloid peptides, 5 μM of Aβ1-42 (test) or Aβ40-1 (control) (Bachem) was added to the reaction immediately before incubation. For reactions involving the addition of fibrillized amyloid peptides, 5 μM of Aβ1-42 or Aβ40-1 was
incubated for 3 days at 37°C prior to being added to the reaction. Substrate levels are expressed as relative fluorescent units (RFU) since a mouse thrombin calibrator and a dedicated thrombin calculating software were unavailable.

Human patient plasma was kindly provided by Dr. Robin Hsiung of the UBC Brain Research Centre in a blinded study. A total of 83 samples were analyzed for thrombin generation, of which 29 patients were diagnosed with AD, 13 were diagnosed with non-AD dementia and 41 were not cognitive impaired controls. In addition to the sex and age of the patient at the time of sampling, known FAD mutations and CSF confirmation of AD diagnoses were also provided. Samples were measured in the same manner as described above, with the exception of the volume of buffer added to reduce CaCl₂ concentrations. Samples were analyzed for the maximum fluorescence as well as times to half-maximal fluorescence. Data were acquired using the Infinite 200 Pro microplate reader (Tecan) and analyzed using Magellan software, version 7 (Tecan).

2.7 Measurement of prothrombin levels

Plasma prothrombin antigen levels were quantified with an enzyme-linked immunosorbent assay (ELISA). Plasma from aged AD and WT mice was isolated via cardiac puncture and prepared as described above. Wells of an Immuno-Sorp plate (Nunc) were coated overnight at 4°C with plasma diluted 1:5 with 0.2 M NaCO₃/Na₂CO₃ buffer, pH 9.6 or serially diluted human thrombin (Sigma) as a standard. After washing three times with washing buffer [PBST (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) containing 0.1%
Tween-20 (Sigma)], samples were blocked with blocking buffer [PBS and 3% bovine serum albumin (BSA)] for 1 hour at 37°C. After a second washing, samples were incubated with an prothrombin detecting antibody (20 µg/mL, Thermo Scientific) in PBST with 3% BSA for 1 hour at 37°C. After washing, bound prothrombin was detected by incubating wells with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) diluted 1:5000 in PBST with 3% BSA for 1.5 hours at 37°C. After washing with washing buffer, samples were developed with 100 µl of TMB Sure Blue (Sigma) for 14 minutes and the reaction stopped with 100 µl of 1 N HCl. Absorbance at 450 nm was measured using a Spectra Max 190 plate reader (Molecular Devices). Plasma samples were analyzed individually (no pooling).

2.8 Measurement of antithrombin activity

Antithrombin (AT) activity was indirectly measured using a Coamatic-Antithrombin kit (Chromogenix), developed to test the functional activity of the enzyme, according to the manufacturer’s instructions. 25 µl of diluted plasma samples and standards (Hemosil) were incubated with heparin in an excess of factor Xa (FXa). A chromogenic substrate (S-2765) mimicking a natural cleavage site of FXa was used to quantify the residual FXa activity, and its absorbance is inversely proportional to AT activity. The reaction was stopped with the addition of 20% acetic acid. The absorbance was read at 405 nm with 490 nm as a reference. Plasma samples were analyzed individually (no pooling). For samples measuring AT activity using amyloid peptides, 50 or 100 µM of Aβ1-42 (test), Aβ1-40 (test) or Aβ40-1 (control) (Bachem) was incubated with 3.74 µg of AT (Haematologic Technologies Inc.) for 24 hours. After
incubation, 25 μl of the reaction mixture was assayed in the same manner as described above. AT activity was also assessed in human patient plasma with the same method as described above. These samples represent the same patients analyzed for thrombin generation and the data was acquired in a blinded manner.

2.9 Antithrombin antigen levels in plasma

AT levels in plasma were semi-quantified by Western blot. One μl of plasma from aged or young AD and WT mice (n=3 for each subgroup) was loaded on to a 10% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose (Pall) according to standard practices. The membrane was blocked with blocking buffer (PBS with 5% skim milk) for 1 hour at room temperature and incubated with a primary rabbit anti-AT (Abcam) antibody diluted 1:1000 in PBST with 5% skim milk overnight at 4°C. After washing three times with PBST, the membrane was incubated with an Alexa Fluor 680-conjugated secondary anti-rabbit IgG antibody (Invitrogen), diluted 1:10 000 in PBST with 5% skim milk and 0.02% SDS in the dark for 1 hour at room temperature. After washing three times, the membrane was developed using the Odyssey Infrared Imaging System (LI-COR).

2.10 Aβ-AT complex detection by Western blot

AT (3.74 μg) was incubated with 50 μM of Aβ1-42, Aβ1-40 or Aβ40-1 at 37°C for 24 hours. The reactions were resolved on a 12% SDS-PAGE gel under reducing conditions and after transferring, the nitrocellulose membrane was treated as described above. Primary antibodies to
mouse anti-Aβ (clone 6E10, Covance) or rabbit anti-AT (Abcam) diluted 1:1000 were used in conjunction with either an Alexa Fluor 680-conjugated secondary anti-mouse or anti-rabbit IgG antibody (Invitrogen), diluted 1:10 000.

2.11 Effect of amyloid peptides on endogenous AT activity

Diluted AD and WT plasma samples were incubated overnight at 37°C with the addition of 5 µg of the peptides Aβ1-42, Aβ1-40 or Aβ40-1. After incubation, the samples were analyzed using the same method as described above to assess AT activity.

2.12 Statistical analysis

All experiments were performed at least three times in duplicate. Statistical comparisons between AD and WT mice were made using Student’s t-test or 2-way ANOVA for unpaired values with Bonferroni post tests where appropriate and analyzed with GraphPad Prism software (version 5, La Jolla, CA). Values are expressed as mean ± standard error mean (SEM) and p-values less than 0.05 are considered significant.
Chapter 3. Results

3.1 Characterization of hemostatic system activation in a mouse model of AD

3.1.1 Whole blood clotting times are similar in AD and WT mice

Previous unpublished studies in our laboratory determined that AD mice had approximately 30% shorter whole blood clotting times, as evaluated through tail bleed assays. Whole blood clotting times are thought to reflect the primary stage of hemostasis, or the formation of the initial platelet plug after injury to the tail veins. It can also reflect small vessel integrity. In an effort to duplicate these results, the same experiment was conducted in a smaller cohort (4 mice of each genotype). Mean bleeding times of aged AD and WT mice were similar (63 ± 15 s and 66 ± 16 s respectively, Figure 3.1) as analyzed by Student’s t-test. These results were unexpected; however, more sensitive assays were later used to detect differences in clotting.
Figure 3.1 Whole blood clotting times of aged AD and WT mice are similar as analyzed by *in vivo* tail bleed assays.

The tails of anaesthetized mice were transected from the tip and the time until cessation of blood flow recorded. Average tail bleeding times are 63 ± 15 s (AD) and 66 ± 16 s (WT). Values represent mean ± SEM, AD n=4 and WT n=4.

3.1.2. Modified prothrombin and activated partial thromboplastin times are faster using AD plasma

The prothrombin time (PT) and the activated partial thromboplastin time (aPTT) are clinical indicators of coagulation, detecting functional abnormalities in the extrinsic or tissue factor pathway and in the intrinsic or contact activation pathway respectively. A modified version of the PT and aPTT was used to distinguish the effects of plasma and/or platelets on clotting. Instead of the classically used platelet poor plasma (PPP), platelet rich plasma (PRP) was isolated from aged AD and WT mice. In addition, platelets from AD mice were resuspended in PPP of WT mice, and the reverse experiment was also performed using platelets from WT
mice in PPP of AD mice. The PT (Figure 3.2A) and aPTT (Figure 3.2B) of AD mice using physiological PRP (32 ± 2 and 67 ± 2 s respectively) were approximately 40% faster compared to those of WT mice (59 ± 6 and 126 ± 7 s respectively). The clotting times of the manipulated or non-physiological PRP were dependent on the type of plasma and not on the type of platelets used. For example, the PT and aPTT of AD plasma with WT platelets (33 ± 2 s, p < 0.01 and 68 ± 2 s, p < 0.001 respectively) had similar clotting times compared to physiological AD PRP. The samples containing WT plasma with AD platelets had similar results (PT of 53 ± 7, p < 0.001 and aPTT of 99 ± 7 s, p < 0.001). These results indicate that the shorter clotting times of AD mice are dependent on AD plasma and the presence of normal platelets, and that AD plasma may contain a prothrombotic component that is interacting differently with platelets.
Figure 3.2 A) The reduced modified prothrombin time (PT) and B) modified activated partial thromboplastin time (aPTT) of aged AD mice are dependent on AD plasma and normal platelets.

The assays were performed in samples containing AD PRP, AD platelets resuspended in WT PPP, WT PRP or WT platelets resuspended in AD PPP. Values represent mean ± SEM, AD n=23 and WT n=19. Statistical comparisons were performed using the Student’s t test. **p < 0.01 and ***p < 0.001.
3.1.3 Platelet activation is similar in AD and WT mice

In order to validate the findings that platelets from AD mice behaved similarly to those from WT mice, platelets were evaluated both in their physiological environment and in isolation. Platelet activation was assessed through flow cytometry using whole blood, platelet rich plasma and isolated platelets. Platelets were also stimulated using thrombin. An antibody against the integrin GPIIb (CD41) was used for platelet identification, while antibodies against P-selectin (CD62P), reflecting α-granule secretion, and the activated conformation of the integrin complex GPIIbIIIa (JON/A), binding fibrinogen leading to platelet aggregation, were used as markers for activation. No significant differences were found during any stages of platelet isolation or activation. Using thrombin as an agonist also did not differentiate platelets from one genotype to another, quantified as the mean fluorescent intensity (MFI) of CD62P expressing cells (Figure 3.3A), MFI of JON/A expressing cells (Figure 3.3.B) and the percent activation or frequency of cells expressing both CD62P and JON/A (Figure 3.3C). These results confirm the previous analyses indicating that reduced clotting times were dependent on the presence of AD plasma and normal platelets. Hematologic profiles previously conducted in our laboratory also reported similar platelet volumes and counts in AD mice compared to WT controls.
A

CD62P expression (MFI)

Unstimulated
Stimulated

AD Platelets
WT Platelets

B

JON/A expression (MFI)

Unstimulated
Stimulated

AD Platelets
WT Platelets
Figure 3.3 Platelet activation is similar in AD and WT mice.

Flow cytometry analysis of platelet activation using two markers of activation, CD62P (P-selectin) and JON/A (GPIIbIIIa). Platelet activation is not significantly different as indicated by A) the mean fluorescence intensity (MFI) of CD62P and B) the MFI of JON/A and C) the percent activation or frequency of CD62P and JON/A expressing cells. Values represent mean ± SEM. Graphs represent the average values of 3 separate experiments.

3.1.4 Thrombin generation is altered in aged AD mice

A limitation to conventional coagulation tests is that plasma can clot when as little as 5% of total thrombin is generated, potentially leaving 95% of remaining thrombin unassayed [92]. To this end, the PT and aPTT can be insensitive in identifying prothrombotic states. Thrombin generation is a functional test which can determine the total amount of thrombin produced as well as provide information about the coagulation system after a clot has formed [92]. It is diminished in factor deficiencies and anticoagulant treatment, while enhanced in inhibitor deficiencies or in an excess of prothrombin.
Thrombin generation was analyzed using a fluorescent substrate for thrombin (Boc-Asp(OBzl)-Pro-Arg-MCA) in plasma of aged or young AD and WT mice. Recalcified plasma was activated using relipidated tissue factor and phospholipids (Innovin) and analyzed for 1 hour. Aged AD mice demonstrated an initial lag in activating the fluorescent substrate in the first 75 s, followed by an increased rate in thrombin generation compared to aged WT mice (Figure 3.4B). The maximum fluorescence of aged AD mice reached more than twice that of aged WT mice (approximately 18 000 vs. 8000 RFU, Figure 3.4A). This may be due to differences in clot turbidity affecting the fluorescent readout. High thrombin concentrations produce less turbid and less permeable clots composed of a denser network of thinner fibers, whereas low thrombin concentrations give rise to more turbid and more permeable clots composed of thicker fibers [93]. In contrast, young AD and WT mice had similar rates of substrate activation and maximum fluorescence levels (Figure 3.5A and B). This indicates that a change is occurring in the plasma of AD mice after 5 months of age, which may be affecting clot turbidity and integrity. Interestingly, young mice of both genotypes reached a higher maximum fluorescence (approximately 11 000 RFU) than aged WT mice—this may alternatively suggest a change may be occurring in WT plasma, where clots are initially comprised of tightly packed, thin fibrin strands and eventually progress to more permeable clots comprised of thicker fibrils.
Figure 3.4 Aged AD mice demonstrate an initial lag in activating a fluorescent substrate for thrombin followed by a large increase in maximum fluorescence compared to WT controls.

A) Thrombin generation profile from 0 to 60 minutes after the reaction start and B) the same thrombin generation profile expanded for the first 5 minutes after the reaction start to emphasize differences in initial rates. Results are pooled for each genotype, AD n=6 and WT n=6.
Figure 3.5 Thrombin generation in young AD and WT mice have similar activation of a fluorescent thrombin substrate and maximum fluorescence.

A) Thrombin generation profile from 0 to 60 minutes after the reaction start and B) the same thrombin generation profile expanded for the first 5 minutes after the reaction start to emphasize differences in initial rates. Results are pooled for each genotype, AD n=4 and WT n=3.
To test if Aβ in the plasma of AD mice was the component imparting the change in initial substrate activation and difference in maximum fluorescence, unfibrillized and fibrillized peptides of Aβ1-42 (test) and Aβ40-1 (control) were incubated with plasma from aged AD and WT mice with varying results (Figures 3.6 and 3.7). Unfibrillized Aβ reflects the composition of diffuse plaques, thought to be preamyloid lesions, whereas fibrillized Aβ of the β sheet conformation comprises the senile or neuritic plaques in the brain [3]. Aβ levels in plasma and CSF decrease with age in AD, reflecting the increasing Aβ deposition in the brain. In the Tg2576 mouse model, Aβ42 plasma concentrations occur in the 500 pM range until 9 months of age, decreasing to the 300 pM range at 18 to 24 months [94]. Using unfibrillized Aβ1-42 and Aβ40-1, activation of the thrombin substrate is dampened by both amyloid peptides in aged AD and WT plasma, accompanied by a slight decrease in maximum fluorescence. In order to test the effect of fibrillization, both peptides were incubated at 37°C for 3 days before being added to AD and WT plasma. In AD plasma, activation of the fluorescent substrate showed slight inhibition by both amyloid peptides, with AD samples eventually achieving similar levels of maximum fluorescence. In WT mice, both amyloid peptides slightly increased the rate of substrate activation as well as maximum fluorescence. Since the effects produced by addition of either unfibrillized or fibrillized amyloid peptides were not specific to the pathogenic or control sequence, the addition of the peptide immediately before the assay does not seem to be a sufficient amount of time for a change to take effect in comparison to the over 18 months the mouse may have been accumulating Aβ in the plasma. Alternatively, Aβ1-42 may not be the sole component in plasma affecting thrombin generation—APP or a cleavage product of a different species may be affecting the reaction instead.
Figure 3.6 Thrombin generation in aged AD and WT mice upon addition of unfibrillized Aβ1-42 and Aβ40-1.

A) Thrombin generation profile from 0 to 60 minutes after the reaction start and B) the same thrombin generation profile expanded for the first 5 minutes after the reaction start to emphasize differences in initial rates. Activation of the thrombin substrate in both AD and WT mice is dampened by both amyloid peptides, accompanied by a slight decrease in maximum fluorescence. Results are pooled for each genotype, AD n=6 and WT n=6.
Figure 3.7 Thrombin generation in aged AD and WT mice upon addition of fibrillized $\alpha$-42 and $\alpha$-40-1.

A) Thrombin generation profile from 0 to 60 minutes after the reaction start and B) the same thrombin generation profile expanded for the first 5 minutes after the reaction start to emphasize differences in initial rates. Activation of the thrombin substrate in AD mice seems to be inhibited by both amyloid peptides, with AD samples achieving similar levels of maximum fluorescence. In WT mice, both amyloid peptides slightly increase substrate activation as well as maximum fluorescence. Results are pooled for each genotype, AD n=6 and WT n=6.
3.1.5 Prothrombin levels in plasma are similar in AD and WT mice

Since thrombin concentrations can alter clot integrity [93], it was of interest to determine whether physiological thrombin concentrations were different between the two genotypes. In addition, data demonstrating an association between increased thrombin accumulation, NFTs and AD pathology have yet to be replicated in the Tg2576 mouse model. The thrombin precursor prothrombin is also implicated in AD, present in astrocytes and microglia of senile plaques [86]. Elevated prothrombin levels are correlated with an increased of arterial and venous thrombosis [95]. However, the thrombotic risk is not due to pathological hemostatic activation but is instead attributed to an increase in thrombin generation, affecting both the initial rate of thrombin generation and the total amount of thrombin produced. In addition, elevated prothrombin levels result in an altered fibrin structure.

Circulating prothrombin levels in plasma were measured through an ELISA in plasma of aged AD and WT mice. Prothrombin levels were similar in both genotypes of mice (Figure 3.8). The mean prothrombin concentrations for AD and WT mice were 148.5 ± 4.8 ng/ml and 143.0 ± 12.0 ng/ml respectively. Although prothrombin antigen levels are similar, it is important to note that the Tg2576 mouse model does not recapitulate the tauopathies and resultant neuronal loss of AD—this may explain in part why concentrations were similar compared to thrombin and prothrombin differences found in human AD patients.
Figure 3.8 Prothrombin levels in plasma are similar in aged AD and WT mice as measured by ELISA.

Mean concentrations of prothrombin in AD and WT mice were 148.5 ± 4.8 ng/ml and 143.0 ± 12.0 ng/ml respectively. Values represent mean ± SEM, AD n=4 and WT n=4.

3.1.6 Antithrombin activity is reduced in aged AD mice

Although prothrombin levels did not differentiate between Alzheimer’s mice and controls, a natural inhibitor of thrombin could potentially be altering the activity of thrombin and affecting clot integrity and subsequent turbidity. Antithrombin (AT) accounts for approximately 80% of thrombin inhibition \textit{in vivo} [96]. The functional activity of AT was indirectly measured through a chromogenic assay in the plasma of aged and young mice (Figure 3.9). Briefly, a mixture of diluted plasma, heparin and a chromogenic substrate was incubated with an excess of Factor Xa (FXa) at 37°C. The cleavage of the chromogenic substrate is inversely proportional to the AT activity, measured as a percentage in the range of 0-125 %. AT activity was reduced in aged AD mice (51.6 ± 4.1 %) compared to WT mice (78.2 ± 8.0 %; Figure 3.9A, p < 0.05) by
approximately one third. In young mice, AT activity was similar in AD and WT mice (116.9 ± 16.5 % and 108.1 ± 18.8 % respectively, Figure 3.9B). These results indicate that AT activity is affected after 5 months of age, and confirm previous findings that a change is occurring in the plasma of AD mice with the progression of age.
Figure 3.9 Antithrombin activity in AD mice is reduced in A) aged mice but not in B) young mice.

AT activity was assessed by an indirect Factor Xa chromogenic assay. AT values in aged mice were significantly lower in aged AD mice (51.6 ± 4.1 %) compared to WT mice (78.2 ± 8.0 %). In contrast, AT activity was similar to young AD mice (116.9 ± 16.5 %) relative to young WT mice (108.1 ± 18.8 %). Values represent mean ± SEM. AD n=3, WT n=3. Statistical comparisons were performed using the Student’s t test. * p < 0.05.
3.1.7 Antithrombin expression in plasma is similar between genotypes

In order to test if reduced AT activity was caused by a lower expression in plasma, AT levels were subsequently measured by Western blot. Protein expression of AT in plasma of aged and young AD and WT were similar in all mice (Figure 3.10) when immunoblotted with an antibody to AT. The unaffected antigen levels combined with the reduced functional activity of AT specific to aged AD mice is reminiscent of type II deficiency, whereby inherited mutations lead to an abnormal AT structure reducing its activity, as well as acquired AT deficiency.

![Western blot analysis of antithrombin expression in plasma of aged and young AD and WT mice.](image)

**Figure 3.10 Western blot analysis of antithrombin expression in plasma of aged and young AD and WT mice.**

AT expression was assessed in plasma using SDS-PAGE techniques and subsequently immunoblotting with an antibody to AT. Protein expression is similar in all samples, with no detectable differences between aged and young mice.
3.1.8 Aβ interacts with antithrombin and lowers its activity in vitro

The interaction between AT and Aβ was measured using the same chromogenic assay for plasma. Purified mouse AT was incubated with synthetic Aβ1-42, Aβ1-40 and the reverse control peptide Aβ40-1 at 37°C for approximately 24 hours. Aβ1-42 and Aβ1-40 reduced AT activity compared to Aβ40-1, and increasing the concentration of Aβ1-42 and Aβ1-40 had an even greater effect (Figure 3.11). 50 μM of Aβ1-42 or Aβ1-40 resulted in a 21.3 % or 16.7 % decrease in AT activity compared to Aβ40-1 (p<0.05). No significant differences were found using 10 μM compared to 50 μM of amyloid peptides. These results indicate an interaction of AT and Aβ occurring independently of clot turbidity on the measurement in plasma. The same reactions were then analyzed by SDS-PAGE and immunoblotted with either an antibody to AT or Aβ. In Figure 3.12A, a slightly higher molecular weight complex was identified above the expected molecular weight of AT (58 kDa) when AT was incubated with Aβ1-40 and blotted with an antibody to AT. These data indicate a potential covalent interaction between Aβ and AT. However, the band identified was quite faint, and may only represent a weak interaction. Alternatively, the peptide interaction may interfere with the binding site for the antibody, producing a weak signal. When the same reactions were blotted with an antibody to Aβ, a band at around 58 kDa was identified when Aβ1-42 was incubated with AT (Figure 3.12B). However, the same band was not present for the interaction between AT and Aβ1-40. The inconsistencies between these results indicate that perhaps the interaction between the two peptides needs to be explored further under different conditions. Native gel electrophoresis may more accurately determine peptide binding between Aβ and AT.
Figure 3.11 Aβ peptides affect AT activity in vitro.

Aβ1-42 and Aβ1-40 peptides decrease AT activity in a concentration-dependent manner, while the reverse peptide Aβ1-40 has little effect on AT activity. Activity is defined relative to the absorbance of AT alone and without AT. 50 μM of Aβ1-42 (81.0 ± 5.2 %) or Aβ1-40 (87.1 ± 2.3 %) results in a respective 21.3 % or 16.1 % decrease in AT activity compared to Aβ40-1 (103.2 ± 2.8 %). Statistical comparisons were performed using the Student’s t test, * p < 0.05. Graph shows mean ± SEM and is a combination of 3 separate experiments.
Figure 3.12 Western blot analysis of AT and Aβ interaction *in vitro*.

A) Immunoblotting with an antibody to AT identified a higher molecular weight complex (identified by the arrow) when AT is incubated with Aβ1-40, indicating a potential interaction.

B) Immunoblotting with an antibody to Aβ, 6E10, identified a band at 58 kDa, the expected molecular weight for AT, when AT is incubated with Aβ1-42.
3.1.9 Amyloid peptides do not differentially affect AT activity in WT plasma

Following the identification of a potential interaction between Aβ and AT, it was of interest to characterize the same interaction using endogenous AT. If the interaction occurs similarly *in vivo*, the addition of Aβ should theoretically normalize AT values to those characteristic of AD mice. However, functional assays were not successful in differentiating between the effects of Aβ peptides and the control peptide. Diluted WT plasma (1:120, as per the manufacturer’s instructions) was incubated at 37°C with the addition of amyloid peptides. All three peptides (Aβ1-42, Aβ1-40 and Aβ40-1) decreased AT activity to a similar degree (Figure 3.13). The incubation of diluted plasma likely caused clotting and unequivocally reduced the turbidity and reported AT activity. This would account for the similar effect of all three peptides in WT plasma. Without incubation, the peptides had no effect at all. In order to accurately assess Aβ binding to endogenous AT in plasma, an assay must be used which does not rely on absorbance to quantify AT activity.
Figure 3.13 Amyloid peptides do not differentially reduce AT activity in WT plasma.

AT activity is reduced in aged AD mice compare to WT controls. Upon incubation with peptides Aβ1-42, Aβ1-40 and Aβ40-1, AT is reduced in WT plasma irrespective of the sequence. Statistical comparisons were performed using the Student’s t test. Graph is representative of 3 experiments. AD n=3, WT n=3.
3.2. Assessment of coagulation system activation in patients with AD

3.2.1 Patient overview

The differences in clot integrity and AT activity in the Tg2576 AD mouse model were convincing, but still required translational data representative of AD patients. 83 plasma samples were provided by the UBC Brain Research Centre in collaboration with Dr. Robin Hsiung. These samples were categorized into three groups: AD (29 subjects), not cognitive impaired (41 subjects) and non-AD dementia (13 subjects). Females comprised the majority of patients, representing 51 of the 83 patients. The average age of the patient was 61.8 years, but the demographics widely ranged between and within each group. The average age of AD patients was 68.8 years, with the majority of patients in their sixties (8 patients) and seventies (8 patients). A smaller number of patients were in their fifties (5 patients) and eighties (4 patients). In contrast, the not cognitive impaired controls had an average of 58.6 years and ranged in age from 34.6 to 74.9 years. The highest proportion of patients were either in their fifties or sixties (16 patients each). The average age of the non-AD dementia group was the lowest at 56.4 years. The age distribution varied between 26 and 80 years in age, with the most number of patients in their sixties (5).

AD patients represented almost entirely the sporadic form, with only one confirmed case containing a PSEN1 mutation. The FAD patient is considered 100% certain of their diagnosis, while 9 other AD patients have prior CSF confirmation resulting in a 99% probability of a positive diagnosis. The remainder of the AD patients who are diagnosed clinically are 85% certain of their diagnosis (Dr. Robin Hsiung, personal communications).
Results were initially analyzed to include patients of all ages. However, in an attempt to control for the most amount of variation between groups, data were selected for patients in their sixties. These samples represented the largest number of patients in all three groups. In addition, they may represent a relatively healthy population compared to a more elderly population with additional health complications. Experiments were initially performed blinded to the diagnoses of patients.

### 3.2.2 Thrombin generation in AD patients results in normal maximum fluorescence and a trend towards faster activation of a thrombin substrate

Thrombin generation was assessed as previously described. Values for maximum fluorescence and times to half maximal fluorescence were used as markers for changes in clot integrity and formation respectively. The fluorescence can be indicative of turbidity and therefore changes in the fibrin network. Clotting times are often assessed as times to half maximal turbidity and conversely lysis times as times from maximal turbidity to half maximal turbidity. Average maximum fluorescence values of AD patients (4414 RFU) were similar compared to not cognitive impaired patients (4506 RFU) (Figure 3.14). Non-AD dementia patients had the highest maximum fluorescence values (4723 RFU). However, these differences were not statistically significant. These data indicate that the final concentration of the thrombin substrate was similar across all samples, and that changes in clot integrity were not affecting the fluorescent readout in contrast to the mouse data.
Times to half-maximal fluorescence were specific to each group, with a trend towards not cognitive impaired patients experiencing the longest times to half-maximal fluorescence (155.9 s) (Figure 3.15). AD patients displayed slightly shorter times to half-maximal fluorescence (146.4 s) and non-AD dementia patients experienced the shortest times (115.1 s). This indicates that the thrombin substrate was initially activated at a faster rate in AD and non-AD dementia patients compared to controls. However, the times displayed a wide variation between samples and as a result, were not statistically significant as analyzed by Student’s t test.
Figure 3.14 Maximum fluorescence values achieved by the activation of a thrombin substrate are relatively normal in AD patients.

Mean maximum fluorescence values of AD patients (4414 ± 421 RFU) were similar compared to not cognitive impaired patients (4506 ± 199 RFU), while non-AD dementia patients (4723 ± 240 RFU) had slightly higher values (not significantly different as analyzed by Student’s t test.)
Figure 3.15 AD and non-AD dementia patients display a trend towards shorter times to half-maximal fluorescence compared to controls.

AD patients exhibited a slightly shorter activation of a thrombin substrate (146.4 ± 43.9 s) compared to not cognitive impaired patients (155.9 ± 45.4 s). However, non-AD dementia patients displayed the shortest times to half-maximal fluorescence at 115.1 ± 47.1 s. Results are not statistically significant as analyzed by Student’s t test.
### 3.2.3. A trend towards lower antithrombin activity in AD patients relative to controls

AT activity was assessed using the same chromogenic assay as previously described. AT values of AD patients (82.2%) were lower relative to not cognitive impaired patients (103.0%) and non-AD dementia patients (98.9%) (Figure 3.16). However, the results were not statistically significant as analyzed by student’s t test comparing one group to another. AT values are known to widely range within the healthy population, and therefore the trend towards lower AT activity is encouraging given the heterogeneity inherent within each group. The differences between the significant reduction in AT activity in the Tg2576 mouse model compared to the human data may be attributed to the familial versus sporadic forms of AD. AT activity has specifically been analyzed for patients in their sixties, and these patients entirely represent the sporadic form of AD.

The reduction in AT activity may be specific to the overproduction of Aβ or an increase in Aβ species prone to aggregation. Though the clinical manifestation of FAD mirrors that of LOAD, the diseases may differ in origin. However, a potential biomarker must also translate to the sporadic form for which early diagnostics do not exist. AT values can generally exhibit a wide distribution in the healthy population without adverse thrombotic effects. AT alone may not provide a unique diagnostic test but its decrease in the Tg2576 mouse model may still provide important information regarding AD pathogenesis. A study representing the familial form may provide more conclusive results regarding the potential of AT as a diagnostic marker.
Figure 3.16 AD patients display a trend towards lower AT activity compared to not-cognitive impaired controls and non-AD dementia.

The average AT activity is 82.2 ± 14.3% for AD patients, 103.0 ± 3.5% for not cognitive impaired patients and 98.9 ± 17.8% for non-AD dementia patients. The results were not statistically significant as analyzed by Student’s t test. p=0.0845 comparing AD and not cognitive impaired patients.
Chapter 4. Discussion

4.1 Accelerated clot formation and thrombin generation affecting clot integrity in Tg2576 mice

Clotting analysis of the Tg2576 mouse model revealed a tendency towards a prothrombotic state. Modified PTs and aPTTs were dependent on AD mouse plasma and the presence of normal platelets. Both assays were similarly affected, with AD mice clotting 40% faster compared to wild-type controls. Other studies conducted in the TgCRND8 AD mouse model demonstrate normal aPTT times using PPP, monitored via the change in absorbance [47]. However, the presence of platelets can greatly impact clot formation and structure. Platelets provide not only a surface on which factors can assemble but are also required for the generation of thrombin. In addition, the classical extrinsic and intrinsic pathways originally described by Davie and Ratnoff [97] as well as Macfarlane [98] have evolved to now include platelets in a cell-based model of coagulation [66]. Including platelets in the modified PTs and aPTTs may provide a more comprehensive view on coagulation in vivo.

The consistency between both assays indicated an abnormality in the common pathway as opposed to deficiencies in the individual intrinsic or extrinsic pathways. Although factors V, VIII and X might also have been affected, thrombin generation was evaluated first. The developing thrombin concentration not only affects the conversion of fibrinogen to fibrin but also the activity of other proteases through various feedback loops inherent in the coagulation cascades.
Prothrombin expression was similar in both AD and WT mice; however, thrombin activity differed between the two genotypes.

Thrombin generation assays revealed differences in initial thrombin concentrations, with aged AD mice demonstrating an initial lag phase in activating the fluorescent substrate followed by an accelerated rate of thrombin activation compared to WT controls. In addition, aged AD mice obtained a maximum fluorescence of more than twice that of aged WT mice. However, the differences in thrombin generation were subject to age, with young mice displaying similar rates of substrate activation and levels of maximum fluorescence. At 5 months of age, Tg2576 mice exhibit a compromised BBB but do not exhibit cognitive or behavioural deficits [44]. Clot turbidity may be affecting the fluorescent readout in aged AD mice. Although fluorescent substrates are less susceptible compared to chromogenic substrates, they can still be influenced by turbidity or aggregation.

High thrombin concentrations produce less turbid and permeable clots composed of a dense network of thin fibers, whereas low thrombin concentrations give rise to more turbid and permeable clots composed of thick fibers [93]. Increased thrombin concentrations are also associated with an increase in clot stiffness, or a decrease in elasticity. However, viscoelasticity is also influenced by other factors including fiber thickness, length, density, branching and cross-linking. Dense, stable clots are associated with thrombosis, while unstable clots are associated with bleeding disorders. Fibrin structure also affects fibrinolysis, with thinner fibers resulting in slower plasmin generation. This can be attributed to a decrease in pore size in the fibrin network limiting access to fibrinolytic enzymes, as well as an increased number of fibers per volume clot.
Thrombin also activates factor XIII, and an increase in its activity can result in a hyperstabilized clot [95].

In support of these data, studies demonstrate an altered clot structure and an inhibition of the tPA-plasmin system in AD both in vitro and in vivo [100]. Aβ-intercalated clots are composed of thinner fibrin strands arranged in a tighter network resistant to fibrinolysis. The viscoelasticity may also be affected, whereby Aβ induces less flexible fibers. In addition, Aβ reduces the turbidity of clots in a concentration-dependent manner [47]. Aβ42 binds to fibrinogen through its C-terminal β-chain and interferes with the binding of plasminogen to fibrin, impairing tPA-mediated plasmin activation [88]. However, obstruction of the plasminogen binding site is independent of fiber thickness. Aβ exposed to pre-formed fibrin monolayers inhibits plasminogen binding and plasmin generation but does not alter fibrin structure. This indicates that Aβ can be incorporated into both preformed and developing clot structures. However, Aβ does not directly inhibit fibrinolytic activity—Aβ and other β-sheet peptides actually stimulate tPA-mediated conversion of plasminogen to plasmin in vitro. This occurs through Aβ acting as a fibrin analogue, and its stimulatory effect increases with aggregated fibrillar forms of Aβ [101, 102]. In vivo, tPA and plasmin activity is downregulated whereas PAI-1 levels are correspondingly upregulated in transgenic mouse models of AD [89, 90]. Injection of Aβ into hippocampal regions in tPA−/− and plasminogen−/− mice also stimulates PAI-1 expression. The difference between the in vitro and in vivo effects of Aβ on the tPA-plasmin system may be attributed to its fibrillar state.
Soluble, oligomeric forms of Aβ are increasingly thought to be more pathogenic compared to aggregated, fibrillar forms of Aβ. In addition, soluble Aβ concentrations correlate well with the severity of dementia in AD [103, 104]. They are present in the frontal cortex of AD patients in concentrations 12-fold higher compared to age-matched controls [105]. Prefibrillar forms of Aβ range in size from dimers to 24-mers, inhibiting long-term potentiation and enhancing long-term depression. They also impair synaptic plasticity and memory loss in vivo. The concentration of the Aβ42 dodecamer, Aβ*56, correlates with memory loss in the Tg2576 mouse model [104]. However, other cognitive and behavioural deficits also appear before Aβ*56 can be detected, suggesting the oligomer may not initiate memory impairment alone. The mechanism by which Aβ oligomerization occurs remains to be elucidated.

### 4.2 Reduced antithrombin activity in Tg2576 mice

Although prothrombin expression was normal in AD mice, an inhibitor may alternatively be affecting free thrombin concentration. Deficiencies in the TFPI, AT and APC pathways all result in enhanced thrombin generation and can also affect fibrin formation. AT lowers the apparent thrombin concentration, thereby producing thicker fibrin strands [93]. As the major physiological inhibitor of thrombin, AT activity was examined through an indirect chromogenic assay. Aged AD mice had significantly lower AT activity compared to age-matched controls. This marker was also subject to age, with young AD mice exhibiting similar levels of AT activity relative to WT mice. These data confirm a change in clot integrity or environment as AD progresses. However, AT expression was normal in both aged and young AD mice.
Congenital and acquired AT deficiencies also exhibit reduced functional AT and are associated with an increased risk for thromboembolic disease. The incidence of AT deficiency ranges from 1 in 600 to 1 in 5000 of the healthy population [106]. Type I AT deficiency results from a range of heterogeneous mutations and is characterized by a reduction of 50% in both antigen and functional activity [107]. In contrast, type II AT deficiency occurs when approximately half of AT function is reduced due to 1) reactive site mutations which disrupt inhibitor-protease interactions 2) heparin binding site mutations which affect heparin cofactor activity or 3) pleiotropic mutations which result in multiple functional defects. Acquired AT deficiencies can result from impaired synthesis or consumptive coagulopathies. Disseminated intravascular coagulation is associated with sepsis and results in the pathological activation of coagulation and deposition of fibrin. It is also characterized by enhanced platelet activation and thrombin generation. The interaction between αIIbβ3 and fibrin results in an altered fibrin architecture resistant to lysis. The procoagulant activity of platelets can also be attributed to the release of polyphosphate (polyP).

PolyP initiates the intrinsic pathway of coagulation through factor XII activation, leading to the generation of the inflammatory mediator bradykinin [69]. Contact system-mediated release of kinins is also associated with E. coli-induced sepsis. In addition, factor XII is a component of senile plaques in AD and in combination with Aβ can stimulate bradykinin generation [108, 109]. PolyP also contributes to proinflammatory responses through platelet-induced capillary leakage [69].
PolyP increases fibrin formation through mechanisms involving factor V, TFPI and TAFI. The prothrombotic role of polyP also extends to clot structure. Clots formed in the presence of polyP are composed of a dense fibrin network with reduced turbidity [110]. The structural changes impede the exposure of C-terminal lysines and the binding of plasminogen and tPA to partially lysed fibrin. This renders the clot resistant to lysis, similar to the effect of Aβ. PolyP is also able to bind both fibrinogen and fibrin. Given the similarities between the effects of polyP and those observed with Aβ, polyP may be contributing to the prothrombotic and proinflammatory responses in AD. Its release upon injury to the vasculature may facilitate enhanced thrombin generation, reduced AT activity and changes in clot integrity.

4.3 Potential interaction between Aβ and antithrombin

There is a dose-dependent effect of Aβ on AT activity in vitro. Synthetic peptides Aβ1-42 and Aβ1-40 reduced AT activity compared to the reverse peptide Aβ40-1. 50 μM of Aβ1-42 or Aβ1-40 resulted in a respective 21.3 % or 16.7 % decrease in AT activity relative to Aβ40-1. The results indicated a potential interaction between Aβ and AT in a buffer system, without the compounding effects of clot turbidity. In addition, Western blot analysis of the same reactions using an AT antibody revealed a slightly higher molecular weight complex between Aβ1-40 and AT, which had a significant effect on AT activity in vitro. The reverse analysis using an Aβ antibody detected a band of a slightly higher molecular weight than AT (58 kDa) in addition to 4 kDa Aβ. Under SDS-PAGE reducing conditions, these data indicate a potential covalent interaction between Aβ and AT. The interaction may need to be explored using more optimal
conditions such as native gel electrophoresis, explaining the inconsistencies between the different species of Aβ.

AT may be interacting with Aβ through a similar mechanism to which it inhibits factors IX, Xa or thrombin. AT requires heparin as its cofactor for its allosteric activation. Heparin, its analogue heparan sulfate and other glycosaminogylcans bind to AT through negatively charged carboxyl and sulfonyl group-containing pentasaccharides [73]. These acidic sequences bind to the lysine and arginine-rich heparin-binding domain of AT, inducing a conformational change that increases its activity by 1000-fold. As a result, a critical arginine residue is brought in close proximity to AT’s reactive site and to a serine residue in the active site of thrombin or other serine proteases, forming a 1:1 covalent complex. Thrombin inhibition is specific in that it requires heparin simultaneously bound to both the serine protease and AT. The ternary bridging complex is facilitated by full-length glycosaminoglycans. In contrast, inactivation of other proteases including factor Xa does not necessitate the same type of inactivation. AT could also bind Aβ in a similar manner to either thrombin or factor Xa. It is also conceivable that AT also requires heparin as a cofactor for binding Aβ, and the addition of heparin into the in vitro system may augment the effect on AT activity.

Both APP and Aβ bind heparin with varying effects. The heparin-binding domain of APP is located adjacent to the C-terminal α-secretase cleavage site, rendering it uniquely part of APPs-α. It is thought to modulate the neuroprotective role of APPs-α, with heparinases reducing neuronal survival in vitro [111]. In contrast, heparin binding of Aβ occurs within residues 12 to 17 and is dependent on its aggregated state [112]. Binding of heparin to fibrillar forms of Aβ
results in an increase in fibril formation and stability. The CAA autosomal dominant HCHWA-D mutated form of Aβ forms fibrils more readily and as a result exhibits increased heparin binding. In addition, amyloid plaques contain glycosaminoglycans. A glycosaminoglycan mimetic was used in a phase III AD clinical trial and though it reduced Aβ aggregation and promoted its clearance, it failed to impact cognitive decline [113]. Low molecular weight heparins (LMWHs) have also been implicated in ameliorating AD pathology. Prophylactic treatment with LMWH enoxaparin in a transgenic AD mouse model reduces neocortical amyloid deposits, total Aβ brain concentration and reactive astrocytosis [114]. Enoxaparin may interfere with heparin-Aβ interactions, reducing amyloidosis and protecting against the toxicity of soluble Aβ.

APP and/or Aβ may be interacting with heparans in vivo and, as a result, may be directly or indirectly affecting AT activity. Heparan sulfate proteoglycans (HSPGs) expressed on the surface of endothelial cells may be facilitating direct interaction of APP/Aβ and AT through a similar mechanism to which AT binds thrombin or factor Xa. Consequently, AT is sequestered from the inhibition of other proteases, decreasing its measurable activity. This would also account for the reduction in the neuroprotective role of APPs-α. Alternatively, the interaction may also be indirect. HSPGs may bind APP/Aβ, reducing the availability of heparan sulfates for the allosteric activation of AT. The negatively charged repeating pentasaccharide sequence is present in only 1% of all vessel-wall heparan sulfate molecules [74]. Indirect inhibition of AT would also decrease its functional activity.

AT may impact the oligomerization and aggregation of Aβ as it confers protection against Aβ-induced toxicity in vitro [115]. Related serine protease inhibitors α1-antichymotrypsin (ACT)
and α1-antitrypsin also induce a similar effect, though their mechanism of action may not involve a reduction in amyloid aggregation. In one study, inhibition is instead attributed to interference with a non-specific serine protease which also protects from the toxicity of amylin, an islet cell specific amyloid [115]. Alternatively, ACT may confer protection against Aβ-induced toxicity through formation of a SDS-stable complex with Aβ [116]. The N-terminal domain of Aβ contains sequence similarity to the active site of serine proteases and binding of this region to ACT promotes destabilization of fibrils in vitro. However, the protective role of AT may differ in its effect on the aggregation state and complex formation with Aβ.

AT has been controversial in its association with AD pathology. In one study, AD stains both amyloid plaques and NFTs in the neocortical and hippocampal regions of AD patients [117]. The intensity of AT staining also corresponds to the severity of pathology. This may be related to the association of thrombin with amyloid plaques and NFTs. AT may have a neuroprotective role in mitigating the effect of the extravasation of thrombin across a compromised BBB. However, the association between AT and AD pathology has yet to be replicated and other findings have instead reported no association of AT with either plaques or tangles [118, 119].

The reduction in AT activity in the present study may also have other functional consequences. AT is a potent antiangiogenic, inhibiting endothelial cell proliferation, blood vessel growth and tumour size in vitro and in vivo [120]. The heparin-binding domain of AT is critical in modulating its antiangiogenic activity. Previous studies in our laboratory have determined that angiogenesis results in a compromised BBB leading to an increase in vascular
density [44]. The reduction in AT activity in aged AD mice may be connected to the increase in angiogenesis, which also occurs after 5 months of age. In addition to its antiangiogenic properties, AT also has roles in modulating inflammation and cell signalling including increasing protacyclin formation in endothelial cells and inhibiting chemokine-induced neutrophil migration [121].

It is also important to acknowledge that the observations made in regard to AT activity in AD mice may not only apply to AT—other anticoagulant pathways including TFPI and APC may also be affected. Further exploration of other endogenous anticoagulants may reveal other deficiencies which also lead to altered clot formation and stability.

### 4.4 Thrombin generation and antithrombin activity in AD patients

A blinded study was conducted using 83 samples of patient plasma, categorized as AD, not cognitive impaired and non-AD dementia. Though the average age of each group was similar, there was a wide age distribution within each group. In order to control for age, data was analyzed specifically for patients in their sixties. These patients represented the highest number of samples in all three groups, and may be relatively healthy compared to a more elderly population. AD patients in their sixties represented entirely LOAD, and the alterations in thrombin generation and AT activity observed in the FAD mouse model may be attributed to a difference in etiology. Maximum fluorescence values obtained in the thrombin generation assay were similar in AD and not cognitive impaired patients. Non-AD dementia patients had slightly higher values, though not significantly different. This was indicative of a normal final
concentration of the fluorescent thrombin substrate in AD samples. In contrast with values observed in AD mice, turbidity did not seem to affect fluorescent readout. This may implicate an unchanged clot integrity in AD patients compared to controls. The times to half-maximal fluorescence were specific to each group, with non-AD dementia patients experiencing the fastest times to half-maximal fluorescence, followed by AD patients. Not cognitive impaired patients displayed the longest times to half-maximal fluorescence, though these results were not significant. The trend towards slightly faster times to half-maximal fluorescence indicates faster activation of the thrombin substrate and may also reflect the faster modified PT and aPTT results performed in AD mouse PRP.

AT in AD patients also displayed a trend towards lower activity compared to not cognitive impaired patients and non-AD dementia patients. However, the reduction in AT activity compared to not cognitive impaired patients (20.8 %) and non-AD dementia patients (16.7 %) was not statistically significant due to a wide distribution of values, and may be attributed to the heterogeneity inherent in the human population. The decrease in AT may also be specific to the Tg2576 mouse model. Perhaps the interaction between AT and Aβ is dependent on the ability of Aβ to oligomerize, the main biochemical aspect of FAD. Alternatively, the interaction may not be unique to Aβ species prone to aggregation. The Swedish mutation does not specifically increase Aβ42 and Aβ40 levels, instead raising the concentration of all Aβ species which may not be representative of the sporadic form of AD. Conversely, AT may be interacting with soluble Aβ species specific in AD mice. A dodecamer similar to Aβ*56 present in the Tg2576 mouse model has not been observed in human cortical extracts, although smaller dimers do impair synaptic function [103].
When interpreting the data observed in Tg2576 mice, it important to acknowledge the limitations of a transgenic mouse model. The overexpression of APP resulting in the increase of all Aβ species and other metabolites leads to amyloid deposition and cognitive deficits but without accompanying neuronal loss. Compounding the difficulty is the difference in etiology of the familial versus the sporadic form of AD. The differences in thrombin generation and AT activity observed in to the Tg2576 mouse model were not successful as a potential diagnostics. However, the translational impact of altered clot integrity and anticoagulant activity may still be relevant in understanding AD pathogenesis.
Chapter 5. Conclusions and Future Directions

5.1 Conclusions

The contribution of vascular dysfunction in AD is an area increasingly gaining exploration. The amyloid cascade hypothesis has primarily served to describe the biochemical changes associated with FAD. However, LOAD has a complex and multifactorial etiology which challenges the traditional dogma. The association of vascular risk factors and related disorders with the development of AD has provided an alternative explanation for disease pathogenesis, though the mechanism by which Aβ may induce cerebrovascular abnormalities remains unclear. A prothrombotic state is increasingly implicated in AD, wherein clot formation, structure and lysis are altered.

The majority of studies described in this thesis use the Tg2576 mouse model, recapitulating the pathology based on the familial form of AD. Modified clotting times were faster using AD plasma compared to WT controls, and were dependent on the presence of normal platelets. Deficiencies in the common pathway were next explored, and thrombin generation exhibited large differences in maximum fluorescence in aged AD mice. The differences in turbidity affecting fluorescence may be attributed to a change in clot integrity, with AD clots composed of a dense network of thin fibrin strands. Changes in the fibrin architecture can also be reflective of reduced anticoagulant activity. AT activity was correspondingly decreased specifically in aged AD mice, implicating an age-dependent change
in clot integrity and anticoagulant activity. The interaction between Aβ and AT was assessed in vitro, displaying a reduction in AT activity in a concentration dependent manner.

Prothrombotic markers were next evaluated in a cohort of patients of three diagnoses: AD, not cognitive impaired and non-AD dementia. The wide age distribution was controlled for by selectively analyzing patients in their sixties. Thrombin generation was assessed using values of maximum fluorescence and times to half maximal fluorescence, indicative of clot integrity and formation respectively. Maximum fluorescence values were similar for all patients, which differed compared to the values observed in mice. In contrast, times to half-maximal fluorescence were group specific, with non-AD dementia patients exhibiting the fastest times to half-maximal fluorescence. AD patients also displayed faster times to half-maximal fluorescence compared to not cognitive impaired controls, though to a lesser extent. AT activity was also analyzed, with AD patients displaying a 20.8% reduction compared to not cognitive impaired controls and a 16.7% reduction relative to non-AD dementia patients. However, these differences were not statistically significant due to a wide range in values for each group. All of the AD patients represented the sporadic form of AD, which may account for the differences observed compared to the FAD mouse model.

Although AT activity may not prove to be a useful diagnostic in the clinic, its consistent decrease in the Tg2576 mouse model may still help elucidate AD pathogenesis. We propose a model in which Aβ lowers AT activity either directly or indirectly. Endogenous HSPGs may facilitate the interaction of Aβ with AT in a similar manner to which AT inhibits thrombin or factor Xa, decreasing its measurable activity. Alternatively, Aβ may indirectly inhibit AT. Aβ may bind to HSPGs, reducing the availability of heparan sulfates for the allosteric activation of
AT and decreasing its functional activity. A reduction in AT would result in an increase in angiogenesis, an observation also reported in the Tg2576 mouse model.

The interaction between Aβ and AT requires further exploration, but its decrease concomitant with changes in clot integrity confirms a tendency towards a prothrombotic state in a FAD mouse model. The manner in which these changes translate to the sporadic form resulting in neurodegeneration remains to be elucidated.

### 5.2 Future directions

#### 5.2.1 Characterization of the Aβ-AT interaction

The interaction between Aβ and AT can be examined through several different methods. Co-immunoprecipitations with Aβ and AT could identify a direct interaction, and can be optimized if the association proves weak as indicated by previous Western blot analysis.

The association of Aβ with AT can also be explored using a fluorescent substrate conventionally used to characterize heparin binding with AT. TNS (2-(p-toluidinyl)naphthalene-6-sulfonic acid) is a hydrophobic probe which binds weakly to AT [122]. Upon excitation at 330 nm, TNS emits a characteristic fluorescent intensity measured at 432 nm. The addition of heparin results in the dissociation of the AT-TNS complex, decreasing the fluorescence by approximately 50 to 60 %. The same assay could be modified to measure the effect of Aβ in the reaction. Aβ could be titrated in the presence of heparin, and a greater decrease in fluorescent intensity would indicate Aβ-AT complex formation. Association and dissociation constants could also be determined from this type of analysis.
Alternatively, tryptophan fluorescence can also be used to characterize the same interaction. AT contains 4 tryptophan residues and their intrinsic fluorescence upon excitation at 280 nm can be exploited to determine heparin affinity. Heparin binding induces a conformational change in AT, increasing the fluorescent intensity by approximately 40%. The addition of Aβ peptides could further enhance fluorescence if the formation of an Aβ-AT complex alleviates the native quenching of tryptophan residues.

The interaction between Aβ, AT and platelets can be explored using AT-deficient plasma. Platelets from either AD or WT mice could be added to AT-deficient plasma and be compared to PRP from AD and WT mice using the PT and aPTT. If clotting times are similar using AT-deficient PRP to those using AD PRP, it would indicate that AT is the component in AD plasma inducing shorter clotting times.

5.2.2 Effect of increasing AT activity in AD mice

The age-dependent reduction in AT activity in Tg2576 mice is concomitant with angiogenesis leading to impaired BBB integrity. In addition to its anticoagulant function, AT is also a potent antiangiogenic, inhibiting endothelial cell proliferation, blood vessel growth and tumour size. Increasing functional AT may reduce hypervascularity and prevent BBB impairment.

AT could be administered both as a prophylactic measure and as a corrective treatment. Low dose AT could be injected intravenously in AD mice as a weekly treatment starting at 3 months of age until an endpoint of 15 months of age. The effect of AT in the circulatory system could be simultaneously monitored using the same chromogenic assay previously described.
Alternatively, AT could also be used as a treatment after angiogenesis and BBB impairment has already occurred. AT could be administered at 11 months of age until and 15 months of age. After the completion of both experiments, mice would be subject to the same coagulation tests previously described including modified PT and aPTT, thrombin generation and AT activity. In addition, angiogenesis and BBB damage could be assessed using markers for microvessel density (CD105) and tight junction morphology (ZO-1) respectively.
References


