Amyloid diseases at old age

A pathological, epidemiological, and genetic study

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ACADEMIC DISSERTATION

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I. Tanskanen M, Peuralinna T, Polvikoski T, Notkola I-L, Sulkava R, Hardy J, Singleton A, Kiuru-Enari S, Paetau A, Tienari PJ, Myllykangas L. Senile systemic amyloidosis affects 25% of the very aged and associates with genetic variation in *alpha2-macroglobulin* and *tau*: a population-based autopsy study. Ann Med 2008 (*in press*).

II. Tanskanen M, Lindsberg PJ, Tienari PJ, Polvikoski T, Sulkava R, Verkkoniemi A, Rastas S, Paetau A, Kiuru-Enari S. Cerebral amyloid angiopathy in a 95+ cohort: complement activation and ApoE genotype. Neuropathol Appl Neurobiol 31:589-99, 2005.

III. Tanskanen M, Kiuru-Enari S, Tienari PJ, Polvikoski T, Verkkoniemi A, Rastas S, Sulkava R, Paetau A. Senile systemic amyloidosis, cerebral amyloid angiopathy, and dementia in a very old Finnish population. Amyloid 13:164-9, 2006.

IV. Tanskanen M, Paetau A, Salonen O, Salmi T, Lamminen A, Lindsberg PJ, Somer H, Kiuru-Enari S. Severe ataxia with neuropathy in hereditary Gelsolin amyloidosis: a case report. Amyloid 14:89-95, 2007.

ABBREVIATIONS

AA	Amyloid A protein
ABri	Amyloid protein in FBD
ACE	Angiotensin converting enzyme
AD	Alzheimer's disease
ADan	Amyloid protein in FDD
AGel	Amyloid protein in hereditary gelsolin amyloidosis
AL	Amyloid protein in Immunoglobulin light-chain associated amyloidosis
AMed	Amyloid protein in aortic medial amyloidosis
APOA1	Apolipoprotein A1
APOE	Apolipoprotein E
Aβ	Amyloid beta protein
BACE2	Beta-amyloid cleaving enzyme2
BBB	Blood–brain barrier
BMI	Body mass index
C	Complement
CAA	Cerebral amyloid angiopathy
CBF	Cerebral blood flow
CNS	Central nervous system
CSF	Cerebrospinal fluid
DM	Diabetes mellitus
ECG	Electrocardiography
EM	Electron microscopy
EMG	Electromyography
FAP	Familial amyloid polyneuropathy
FBD	Familial dementia, British
FDD	Familial dementia, Danish
HCHWA-D	Hereditary cerebral hemorrhage with amyloidosis, Dutch
HCHWA-I	Hereditary cerebral hemorrhage with amyloidosis, Icelandic
HT	Hypertension
IHC	Immunohistochemistry
LCA	Leukocyte common antigen (CD45)
LPL	Lipoprotein lipase
LRP	Low-density lipoprotein receptor-related protein
MI	Myocardial infarction
MRI	Magnetic resonance imaging
MUP	Motor unit potential

PCR	Polymerase chain reaction
PNP	Polyneuropathy
PP	Precursor Protein
PS	Presenilin
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
QST	Quantitative sensory testing
SAP	Serum amyloid P-component
SEP	Sensory evoked potential
SMI311	A pan-neurophilament antibody cocktail
SNP	Single nucleotide polymorphism
SSA	Senile systemic amyloidosis
TTR	Transthyretin

ABSTRACT

This purpose of this study was to investigate the frequency, genetic- and health-associated risk factors, mutual association, and amyloid proteins in three old age-associated amyloid disorders: senile systemic amyloidosis (SSA), cerebral amyloid angiopathy (CAA), and hereditary gelsolin (AGel) amyloidosis. The study was part of the prospective population-based Vantaa 85+ autopsy study on a Finnish population aged 85 years or more (n = 601) and was completed with a case report on a single patient with advanced AGel amyloidosis.

The diagnosis and grading of amyloid were based upon histological examination of tissue samples obtained post mortem and stained with Congo red. The amyloid fibril and associated proteins were characterized by immunohistochemical staining methods. The genotype frequencies of candidate gene polymorphisms and information on health-associated risk factors in subjects with and without SSA and CAA were compared.

In a Finnish population ≥ 95 years of age, SSA and CAA occurred in 36% and 49% of the subjects, respectively. In total, two-thirds of these very elderly individuals had SSA, CAA, or both, however these two conditions co-occurred in only 14% of the population. In subjects 85 years or older, the prevalence of SSA was 25%. In this population, SSA was associated with age at the time of death (p=0.002), myocardial infarctions (MIs; p=0.004), the G/G (Val/Val) genotype of the exon 24 polymorphism in the *alpha2-macroglobulin* (*α2M*) gene (p=0.042), and with the H2 haplotype of the *tau* gene (p=0.016). In contrast, the presence of CAA was strongly associated with *APOE* ε4 (p=0.0003) and neuropathological AD (p=0.0005), and with clinical dementia (p=0.01) in both ε*4*+ (p=0.02) and ε*4*- (p=0.06) individuals. Apart from demonstrating the amyloid fibril proteins, complement proteins 3d (C3d) and 9 (C9) were detected in the amyloid deposits of CAA and AGel amyloidosis, and α2M protein was found in fibrotic scar tissue close to SSA.

In conclusion, the study shows that while SSA and CAA do not associate with each other, the occurrence of one or both of them is extremely common in elderly individuals. Old age, MIs, the exon 24 polymorphism of the *α2M* gene, and H1/H2 polymorphism of the *tau* gene associate with SSA, .while clinical dementia and *APOE* ε4 genotype associate with CAA. The high prevalence of CAA, combined with its association with clinical dementia independent of *APOE* genotype, neuropathological AD, or SSA, also highlights its clinical significance in very elderly individuals, in whom the serious end stage complications of CAA, namely multiple infarctions

and hemorrhages, are rare. Further studies are warranted to confirm the findings in other populations and to clarify the role of α2M and *tau* in the pathogenesis of SSA. Importantly, the role of complement in amyloidosis should be further investigated, in particular its involvement in the process of amyloid beta (Aβ) protein elimination from the brain. Finally, the high prevalence of SSA in the elderly raises the need for prospective clinical studies to define its clinical significance.

INTRODUCTION

Along with the increased life span of individuals (http://www.un.org/esa/socdev/ageing/agewpop.httm), the burden of old age-associated diseases has inevitably increased (Jagger et al., 2007). Alzheimer's disease (AD), probably the most well-known geriatric disease, belongs to the old age-associated amyloid diseases. While AD affects millions of elderly individuals in the Western countries (Gorshow, 2007) and is included as one of the leading causes of death (Heron and Smith, 2007), other representatives such as cerebral amyloid angiopathy (CAA) and senile systemic amyloidosis (SSA) have attained much less medical, and public, attention.

The term "amyloid" refers to the precipitation of protein in tissue, mainly as extracellular depositions of protein fibrils and with a characteristic appearance in electron microscopy, a typical X-ray diffraction pattern, and an affinity for Congo red with concomitant green birefringence (Westermark et al., 2005). Virchow introduced (Virchow, 1854a,b), the term "amyloid" to the medical literature in 1854 to describe cerebral cortical red homogenous material, and since then knowledge about amyloid composition has accumulated and a long list of amyloid diseases are now recognized. This study presents pathological, epidemiological, and genetic data for three forms of age-associated amyloid disorders, specifically SSA, CAA, and AGel amyloidosis.

REVIEW OF THE LITERATURE

1. AMYLOID

1.1. The term "amyloid" – historical aspects

The term "amyloid" was coined in the year 1838 by the German botanist Matthias Schleiden to describe a normal amylaceous constituent in plants (Kyle, 2001). In the medical literature, the term was used in 1854 for the first time by Virchow (Virchow, 1854a) who noted that small round deposits in the nervous system showed the same color reaction with iodine and sulfuric acid, a change from brown to blue which was typical for starch. Based on this, he was convinced that these structures were identical to starch (Virchow, 1854b) and named them "corpora amylacea" after the Latin term "amylum" (starch). In contrast to Virchow, representatives from the French and British Edinburgh schools thought that amyloid was more closely related to cellulose than starch. They preferred to use the terms "lardaceous" (based on the bacon-like appearance of the tissue) or "waxy" (based on the homogeneity of the material). These schools also used the term "sago" (a sweet substance in certain palm species) to describe a spleen, in which the follicles were converted into the waxy material.

Notable milestones in the history of amyloid after its first account by Virchow are listed in **Table 1**. A new insight into the biochemical character of amyloid was attained in 1859 when Friedrich and Kekule (Kyle, 2001) reported a high proportion of nitrogen in amyloid-infiltrated organs. This led to the idea that amyloid was composed of proteins instead of carbohydrates. Virchow never agreed with this theory, which is now known to be correct. In 1875, further progress took place in the diagnostics field when Cornil in Paris, Heschl in Vienna, and Jürgens in Berlin independently described the usefulness of methylviolet staining to detect amyloid, compared to Virchow's iodine sulfuric acid test (Kyle, 2001). Methylviolet is a "metachromatic" stain, a term introduced in 1878 by Ackroyd and Ehrlich to describe the staining reaction of amyloid (Kyle 2001). Nevertheless, Virchow rejected the metachromatic stains of amyloid as long as 10 years after their discovery. However, the metachromatic stains were helpful in 1876 in detecting amyloid in heart tissue (Soyka J. Prag Med Wschr 1: 165, 1876; cited in Hodgkinson [Hodgkinson and Pomerance, 1977] and Buerger [Buerger

and Braunstein, 1960], **Table 1**). Eventually, metachromatic staining was replaced by Congo red. In 1907, Alzheimer described "senile" plaques and neurofibrillary tangles in a demented patient (Alzheimer et al., 1907, **Table 1**). Later, in 1927, Divry reported amyloid material within senile plaques in Alzheimer's disease (Divry P. Etude histo-chimique des plaques seniles. J de neurologie et de Psychiatrie 27: 643-657, 1927; cited in Kyle [Kyle, 2001]). In 1954, cerebrovascular amyloid was described by Pantelakis (Pantelakis S. A particular type of senile angiopathy of the central nervous system: congophilic angiopathy, topography and frequency [Article in French]. Monattsschr Psychiatr Neurol 128:219-256, 1954).

Table 1. Notable years in the history of amyloid and amyloid diseases, with special focus on SSA, CAA, and AGel amyloidosis.

Year	Event
1854	Virchow introduces the term "amyloid" into the medical literature
1859	Friedrich and Kekule report on nitrogen in amyloid deposits
1876	Soyka describes cardiac amyloid
1884	Böttiger creates Congo red dye
1907	Alzheimer et al. describe senile plaques and neurofibrillary tangles
1922	Bennhold notes that Congo red binds to amyloid
1927	Divry et al. describe Congo red in senile plaques
1954	Pantelakis introduces the term "congophilic angiopathy"
1959	Cohen and Calkins describe the fibrillar ultrastructure of amyloid in EM
1962	Puchtler introduces a new modification of Congo red
1968	Eanes and Glenner describe the secondary (X-ray diffraction) structure of amyloid
1968	Pras introduces a method to extract proteins from fibrils
1979	Meretoja describes Finnish type (AGel) amyloidosis
1980	Sletten et al. detect prealbumin (TTR) as the main amyloid fibril protein in "senile cardiac amyloidosis"
1984	Pitkänen et al. introduce the term SSA
1984	Glenner and Wong describe cerebrovascular Aβ protein
1990	Ghiso et al. and Maury et al. describe variant gelsolin as an amyloid protein in AGel amyloidosis
1995	Gustavsson et al. propose that TTR fibrils are composed of wildtype protein in SSA

SSA = senile systemic amyloidosis; CAA = cerebral amyloid angiopathy;

AGel amyloidosis = hereditary gelsolin amyloidosis; EM = electron microscopy;

TTR = transthyretin.

A substantial advance in amyloid research took place in the late fifties, when the fibrillar ultrastructure of amyloid was discovered using electron microscopy (EM) in 1959 (Cohen AS and Calcins E. Nature 183: 1202-3,

1959; cited in Vinters [Vinters, 1996]). Another advancement was made in 1966 when the pentagonal protein serum amyloid P component was described (SAP; Bladen HA, Nylen MU, Glenner GG. Ultrastruct Res 14: 449-59, 1966; cited in Kyle [Kyle, 2001]). Two years later, a significant step forward in the chemical characterization of amyloid proteins took place when a method to extract proteins from fibrils using water was introduced (Pras et al., 1968), enabling the rapid identification of new amyloid proteins. In 1971, the biochemical basis of AL amyloidosis was unraveled (Glenner et al., 1971). During the subsequent years, AA amyloid was characterized as an amyloid protein in the previously called "secondary" (inflammation-associated) amyloidosis (Benditt EP, Eriksen N. Lab Invest 26: 615-25,1972; cited in Kyle [Kyle, 2001]). In addition, serum amyloid A protein, an acute phase protein, was soon identified in blood (Levin M, Pras M, Franklin EC. J Exp Med 138: 373-80, 1973; cited in Kyle [Kyle, 2001]; Husby and Natvig, 1974). In 1978, prealbumin (transthyretin, TTR) was found to be characteristic for amyloid material in familial Portuguese amyloid polyneuropathy (FAP; Costa et al., 1978); a clinical disease described already in 1951 (Corino de Andrade, M. Preliminary note on an unusual form of peripheral neuropathy. Rev Neurol (Paris) 85: 302-6, 1951; cited in Kyle [Kyle, 2001]). In 1979, Meretoja described Finnish amyloidosis, now known as AGel amyloidosis (Meretoja, 1979). In 1980, TTR was found to be the characteristic protein in "senile cardiac amyloidosis" (SCA; Sletten et al., 1980). SCA was later renamed senile systemic amyloidosis (SSA), in order to emphasize the systemic nature of this wildtype TTR-associated amyloidosis (Cornwell et al., 1983; Gustavsson et al., 1995). In 1983, cystatin-C was found to represent the protein characteristic of amyloid in the Icelandic type of familial cerebral amyloid angiopathy, (HCHWA-I; Cohen et al., 1983) and in the same year, a point mutation in the gene coding for TTR, resulting in a substitution of methionine instead of valine at position 30, was reported for FAP (Tawara et al., 1983). In 1984, Glenner and Wong gave the first report on the AD-associated cerebrovascular Aβ (Glenner and Wong, 1984a) and the following year beta 2 -microglobulin was described to characterize the amyloid in the dialysis-related amyloid arthropathy (Gejyo et al., 1985). In 1988, apolipoprotein A1 (APOA1) was characterized as the amyloid protein in the hereditary amyloid disease in Iowa, USA (Nichols et al., 1988). In 1990, variant gelsolin was described as the amyloid protein in the Finnish form of FAP, now preferentially referred to as hereditary AGel amyloidosis (Ghiso et al., 1990; Maury et al., 1990). Later, several proteins were characterized describing three different familial amyloid diseases showing a preference for renal manifestation: fibrinogen A-α chain (Benson et al., 1993), lysozyme (Pepys et al., 1993) and apolipoprotein AII (Benson et al., 2001).

Major progress has taken place in amyloid research during the last 150 years. It is now clear that the cerebral corpora amylacea are not composed of proteins, but glycogen-like substances with sulfate and phosphate groups

as Virchow claimed. Furthermore, it is also clear that cerebral corpora amylacea do not represent amyloid. Interestingly, the term "amyloid" has still prevailed. The reason for this resides in history, based partly on Virchow's standing as one of the leading pathologists of his time and partly on iodine staining, which was used for a long time as the diagnostic test for amyloid (Doyle, 1988).

1.2. Structure

1.2.1. Congophilia with apple-green birefringence

Congophilia with apple green birefringence (Divry P. Etude histo-chimique des plaques seniles. J de Neurologie et de Psychiatrie 27:643-57, 1927; cited in Sipe [Sipe, 2000]) was the first criterion for amyloid (Sipe, 2000). Congo red dye was invented by the German chemist Böttiger already in the year 1884 (Böttiger P. Deutsches Reich's Patent 28753, August 20, 1884; cited in Frid [Frid et al., 2007]). Congo red is an aniline dye originally used for staining textiles, but is also capable of staining all types of amyloid. Böttiger created the first "direct" dye, which did not require additional substances for fixation to the textile fibers. The name "Congo" was given to the new dye by the owner of the patent, the AGFA Corporation, inspired by a diplomatic conference held in Berlin in 1884-1885 in order to mediate a trade dispute between several European colonial powers in the Congo River Basin in Central Africa (Kyle, 2001; Steensma, 2001). The name was thought to be effective for marketing purposes since "Congo" referred to an exotic place, and moreover, in those days it was on the "tip of the lips" (Kyle, 2001; Steensma, 2001). In addition to staining textiles, Congo red was used to stain tissues already in 1886 (Steensma, 2001), but it was not until 1922 that Bennhold noted its capacity to bind to amyloid (Bennhold H. Eine spezifische Amyloidfärbung mit Kongorot. Münchener Medizinische Wochenschrift (November):1537-1538, 1922; cited in Kyle [Kyle, 2001]). In the year 1962, Puchtler described the renewed method (Puchtler et al., 1962) which is still widely used.

The chemical name of Congo red (also known as "direct red", "direct red 28", or "cotton red") is 3,3′-[(1,1′-biphenyl)-4,4′-diylbis(azo)] *bis*-(4-amino-1-naphtalene acid) disodium salt ($C_{32}H_{22}N_6O_6S_2{\cdot}2Na$). It is a symmetrical molecule with a hydrophobic center and is composed of two phenyl rings. The rings are linked via diazo bonds to two charged terminal naphtalene moieties. The terminal parts of Congo red contain sulphonic acid and amine groups, and it has a molecular weight of 696.7 g/mol and diameter of approximately 21Å (Romhanyi, 1971). Congo red exists in chinone form in acidic solution, and in sulphonazo form in basic solution, changing the color from blue (below pH 3) to red (above pH 5) and can thus be used

as a pH indicator as well. The binding of Congo red to amyloid induces a characteristic shift in the maximal optical absorbance of the molecule from 490 nm to 540 nm. The mechanisms of interaction between Congo red and amyloid fibrils has been intensively studied (Klunk et al., 1989; Turnell and Finch, 1992) but the process is not completely understood (Frid et al., 2007). Generally, it is believed that Congo red binding depends on the secondary, β-pleated configuration of the fibril, and is possibly mediated by hydrophobic interactions of the benzidine centers as well as the electrostatically charged terminal groups (Frid et al., 2007).

The Puchtler modification (Puchtler et al., 1962) of Congo red staining is widely used in pathology as the first step in detecting amyloid in histological specimens, although individual laboratories may apply their own variant of the method. Congo red staining is also applicable to frozen sections and for staining devices. In diagnostics, the formalin-fixed histological samples are generally embedded in paraffin, sectioned 5-8 μm thick (brain samples), stained with Congo red, and viewed in a light microscope under polarized light in which amyloid can be seen as red to green birefringent homogeneous material. Interestingly, the light microscope finding has been recently observed to vary in different types of transthyretin (TTR)-related amyloidosis. Therefore, distribution into two different histological patterns of amyloid deposition (designed as A and B) has been proposed (Bergstrom et al., 2005). In pattern A, seen in SSA and in some TTR-associated FAP cases, amyloid material is noted to have a homogenous, patchy distribution displaying only weak congophilia. In pattern B, detected in part in FAP patients, amyloid appears as thin streaks and is strongly congophilic. After this proposal, the biochemical structure of amyloid fibrils was supposed to be transmitted to the microscopic finding.

1.2.2. Fluorescence microscopy

Amyloid can also be visualized using a fluorescence microscope. A fluorescence microscope is a light microscope used to study the properties of organic and inorganic substances using the phenomena of fluorescence and phosphorescence. The component of interest in the specimen is labeled with a fluorescent molecule called the fluorophore. However, fluorophores, lose their ability to fluoresce as they are illuminated. In fluorescence microscopy, amyloid can be detected using thioflavin stains which emit green fluorescence when they are bound to amyloid. Thioflavin-T (Basic Yellow 1 or CI 49005) is a benzothiazole salt, obtained by methylating dehydrothiotoluidine with methanol in the presence of hydrochloric acid. When the dye binds to β sheets, it undergoes a characteristic 120 nm red shift of its excitation spectrum that may selectively be excited at 450 nm, resulting in a fluorescence signal at 482 nm. Thioflavin-S is a mixture of compounds resulting from the methylation of dehydrothiotoluidine with sulphonic acid. Both thioflavin-T and -S stains can be used to visualize

amyloid. Although the fluorescence method is also specific for amyloid (Revesz et al., 2003), the loss of fluorescence is disadvantageous since the reaction cannot be re-examined later.

1.2.3. Fibrillar morphology

The fibrillar morphology of amyloid, based on the analysis of contrasted tissue sections by EM (Cohen and Calkins, 1959), has been adopted as the second criterion defining amyloid (Sipe and Cohen, 2000). Of the several types of EM, scanning transmission and single particle molecular averaging EM have been considered to be very powerful in modern amyloid research (Lashuel and Wall, 2005). The technique used to prepare the sample material for EM varies depending on the specimen and the analysis required. In order to detect amyloid, the samples are usually dehydrated, embedded with a resin such as epoxy, sectioned into thin slices, and stained using heavy metals such as lead, uranium, or tungsten. The idea of staining is to scatter imaging electrons and to create contrast between different structures, since many materials, particularly biological materials, are nearly transparent to electrons. In biology, specimens are usually stained "en bloc" before embedding, and then stained directly after sectioning by brief exposure to aqueous or alcoholic solutions of the heavy metal stains. In the original work of Cohen and Calkins, the sections were stained with uranyl acetate and lead citrate.

The ultrastructure of amyloid deposits of diverse origins in humans and animals (Cohen A.S. et al, Electron microscopy of amyloid in Harris J.R. (ed.) Electron Microscopy of Proteins, Vol 3, pp. 165-205) consist of straight, rigid fibrils ranging in width from 6-13 nm (10 nm=100Å), on average 7.5 to 10 nm, and a length ranging from 100-1600 nm. Two or more filamentous subunit structures, 2.5 to 3.5 nm in diameter, occasionally crossing each other, longitudinally constitutes this 7.5-10 nm -wide amyloid fibril.

1.2.4. Secondary structure: the pleated beta (β) sheet

The secondary structure, typical to all amyloids, was revealed by X-ray diffraction analysis of isolated amyloid protein fibrils (Eanes and Glenner, 1968; Bonar et al., 1969; Sunde and Blake, 1997).

The initial isolation method of amyloid fibrils was based on a gentle physical separation and homogenization in saline, followed by low-speed centrifugation, yielding a layer of fibrils not present in sedimentation pellets of normal tissues (Cohen and Calkins, 1964) and demonstrating a green birefringence in polarized light after staining with Congo red. Amyloid fibrils isolated from tissues and organs by a physical separation model using sucrose gradient centrifugation (Shirahama and Cohen, 1967) was also equal in morphology to those tissues. Pras introduced the water extraction method (Pras et al., 1968) that has been widely used for extracting almost all biochemical types of amyloid except Aβ and prion protein amyloid (Selkoe

and Abraham, 1986; Prusiner and DeArmond, 1990). With the low ionic strength Pras method, amyloid fibrils are separated from all proteins soluble in physiological saline by extraction of homogenates of amyloid-laden tissues with physiological saline, followed by differential centrifugation. Then, amyloid fibrils can be separated from other saline-insoluble tissue proteins by suspension in water.

In nature, most proteins have both alpha helix and beta (β) sheet secondary structure. In the amyloid form, the proteins are mostly in the β-pleated sheet conformation though not exclusively. Factors influencing changes in the spatial form of proteins include increased protein content, low pH, metal ions and chaperones, proteins that are associated with amyloid deposits but are not part of the insoluble fibrils themselves (see later; Ghiso and Frangione, 2002). The secondary structure of amyloid consists of the polypeptide backbone, mostly in the β-pleated sheet conformation, oriented perpendicular to the fibril axis.

1.2.5. Amyloid fibril proteins and their nomenclature

The modern nomenclature of different types of amyloid (**Table 2**) is based on the amyloid fibril protein. An informal amyloid nomenclature committee was established in 1974 in Helsinki, Finland, in connection with the first Internal Symposium on Amyloidosis. Thereafter, the committee has met several times in order to create the official nomenclature lists for each type of amyloid (Westermark et al., 2005). For the amyloid fibril protein to be included in the official nomenclature list, it must be unambiguously characterized and described in a peer-reviewed paper. According to the present nomenclature, amyloid is formed on extracellular deposits of proteins. Apart from these extracellular fibril proteins, several intracellular protein inclusions have been described, and except for neurofibrillary tangles, these proteins are not included in the amyloid list. The current lists for human (**Table 2**) and animal amyloid fibril proteins have been presented by Westermark et al. (Westermark et al., 2005), and include a separate list of inclusions with aggregated proteins of known biochemical composition, with or without amyloid properties (Westermark et al., 2005).

Table 2. Human amyloid fibril proteins and their precursors.

Amyloid protein	Precursor protein	Type	Syndrome/Involved tissue
AA	(Apo)serum AA	S	Reactive (previously: "secondary")
AANF	Atrial natriuretic factor	L	Cardiac atria
AApoAI	Apolipoprotein AI	S	Familial
		L	Aorta, meniscus
AApoAII	Apolipoprotein AII	S	Familial
AApoAIV	Apolipoprotein AIV	S	Sporadic, aging
ABri	ABriPP	S	Familial dementia, British
ACal	(Pro)calcitonin	L	C-cell thyroid tumors
ACys	Cystatin C	S	Familial
ADan	ADanPP	L	Familial dementia, Danish
AFib	Fibrinogen α-chain	S	Familial
AGel	Gelsolin	S	Familial (previously: "Finnish")
AH	Immunoglobulin heavy chain	S; L	Myeloma-associated (previously: "primary")
AIAPP	Islet amyloid polypeptide (previously: "amylin")	L	Insulinomas, aging Islets of Langerhans
AIns	Insulin	L	Iatrogenic
AKer	Kerato-epithelin	L	Familial Cornea
AL	Immunoglobulin light chain	S; L	Myeloma-associated (previously: "primary")
ALac	Lactoferrin	L	Cornea
ALys	Lyspzyme	S	Familial
AMed	Lactadherin	L	Aortic and arterial media, aging
AOAAP	Odontogenic ameloblast-associated protein	L	Odonogenic tumors
APro	Prolactin	L	Prolactinomas, aging Pituitary gland
APrP	Prion protein	L	Spongiform encephalopathies
ASemI	Semenogelin I	L	Vesicula seminalis
ATau	Tau*	L	Aging, AD, FTL** Intracellular
ATTR	Transthyretin	S	Familial, SSA
		L?	Tenosynovium
Aβ	Aβ protein precursor (AβPP)	L	Aging, AD, CAA
Aβ2M	β2-microglobulin	S; L?	Hemodialysis-associated Joints

S = systemic; L = localized; FTL = frontotemporal dementia; SSA = senile systemic amyloidosis; CAA = cerebral amyloid angiopathy.

*intracellular.

**and other cerebral conditions.

In addition to fibril proteins, amyloid deposits contain other proteins (**Table 3**), which are not part of the insoluble fibrils themselves, called "pathological chaperones" (Ghiso and Frangione, 2002). These proteins include amyloid P component, apolipoprotein E (APOE), apolipoprotein J, glycosaminoglycan perlecan, vitronectin, α1-antichymotrypsin, and complement proteins (Sipe, 1992; Revesz et al., 2003; Buxbaum, 2006).

Table 3. Proteins associated with amyloid fibrils ("pathological chaperones").

Protein	Reference
Amyloid P-component	Pepys et al., 1994
Apolipoprotein E	Gallo et al., 1994
Apolipoprotein J	Yerbury et al., 2007
Complement	Matsuoka et al., 2001
Perlecan	Ancsin and Kisilevsky, 1999
Vitronectin	Eikelenboom et al., 1994
α1-antichymotrypsin	Ma et al., 1994

1.3. Formation of amyloid

The present hypothesis on the process of amyloid formation suggests that the amyloidogenic precursor proteins undergo misfolding, which allows them to populate an immediate precursor pool, from which they rapidly aggregate (Buxbaum, 2004). Depending on the particular protein, several mechanisms are operative and may involve: nonphysiological proteolysis, defective physiological proteolysis, and mutations with changes in thermodynamic or kinetic properties, resulting in oligomeric aggregation. This is followed by the assembly of higher order structures that become insoluble under physiological conditions.

For a long time, fibrils found in tissues were thought to be biochemically and structurally identical to the isolated amyloid protein fibrils used in *in vitro* studies (Sipe and Cohen, 2000). However, dissimilarities have been found between amyloid fibrils in tissue deposits and isolated preparations using high resolution EM (Inoue et al., 1998). In addition, *in vitro* studies suggest that the formation of amyloid fibrils is the result of a combination of factors, not only including the primary structure of the polypeptide, but also the thermodynamic parameters of the environment (Kelly, 1998). Thus, results from *in vitro* studies should only cautiously be applied to *in situ* tissues.

1.4. Genetics and amyloid

In the field of amyloid research, genetic analyses have been focused on two different areas. First, genetic modifications in precursor proteins can influence amyloid fibril formation since point mutations in the coding region of a gene can cause amino acid substitutions, influencing amyloid fibril formation (Revesz et al., 2003). These mutations can influence or alter the rate of conversion of a native protein to the fibrillar form. Examples of this include the amyloidogenic variant of cystatin-C deposited in hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I), or the enhanced amyloidogenic properties of the mutant E22Q Aβ peptide which is associated with hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D). Other possible genetic modifications of precursor proteins resulting in amyloid formation include truncation (e.g. Gerstmann-Sträussler-Scheinkler with YI45STOP of the prion protein) and elongation (e.g. ABri and ADan precursor proteins in the BR12 gene -related dementias) (Revesz et al., 2003). The second role of genetics in amyloid research is in the investigation of possible associations between clinical amyloid diseases and genetic alterations. Well-known examples of this include the multiple mutations in the gene for *TTR* in FAP syndromes, or the established association between the *APOE* ε4 allele and AD.

2. OLD AGE-ASSOCIATED AMYLOID DISEASES

2.1. General aspects

The age-related amyloid diseases (**Table 4**) have come of age (Cornwell et al., 1995). Among them, AD has received the most attention, although during the last two decades several other amyloid diseases manifesting at old age have been identified. In addition, causative fibril proteins have been recognized but common amino acid sequences in the amyloid fibril proteins causing the age-related amyloid diseases have not been identified (Cornwell et al., 1995). The old age-associated amyloid diseases include SSA and amyloidosis associated with AD, including CAA, senile aortic (AMed) amyloidosis, isolated atrial amyloidosis, amyloidosis of the seminal vesicles, amyloidosis of the islets of Langerhans, and amyloidosis of the pituitary gland (Cornwell et al., 1995). Of these, only SSA is a systemic disorder. The others occur in a single localization only, and the fibril protein precursors are synthesized locally in the tissue involved. In SSA, the precursor protein is synthesized mainly in the liver. There are also some age-associated intracellular amyloid

forms, like the neurofibrillary tangles in neurons in AD, the choroid plexus amyloid (Eriksson and Westermark, 1986), and adrenal cortical amyloid (Eriksson and Westermark, 1990). In several hereditary forms of amyloid diseases, symptoms may manifest only in middle age, or even old age, although the genetic defect is already present at birth. In this sense, the hereditary amyloid diseases are also associated with aging.

Table 4. The old age-associated amyloid diseases.

Disease*	Amyloid protein	Reference
Alzheimer's disease	Aβ,	Masters et al., 1985
	Tau**	Grundke-Iqbal et al., 1986a
AMed (senile aortic) amyloidosis	AMed	Haggqvist et al., 1999
Amyloidosis of the adrenal cortex	Inclusion bodies**	Eriksson and Westermark, 1990
Amyloidosis of the choroid plexus	Tau**	Eriksson L and Westermark, 1986
Amyloidosis of the islets of Langerhans	AIAPP	Westermark et al., 1990a
Amyloidosis of the pituitary gland	APro***	Westermark et al., 1997
Amyloidosis of the seminal vesicles	ASemI	Linke et al., 2005
CAA	Aβ	Glenner and Wong, 1984a
Isolated atrial amyloidosis	AANF	Rocken et al., 2005
SSA	ATTR	Sletten et al., 1980

CAA = cerebral amyloid angiopathy; SSA = senile systemic amyloidosis.

*Note: hereditary amyloid diseases are not included in this list.

**Intracellular deposition.

***Occasionally other pituitary hormone.

Apart from age and heredity, amyloid formation may be associated with several other conditions including malignant neoplasms, chronic dialysis, or chronic inflammation such as rheumatoid arthritis. In the following chapters, four forms of age-associated amyloid diseases are presented: SSA, CAA, hereditary AGel-, and AMed amyloidosis.

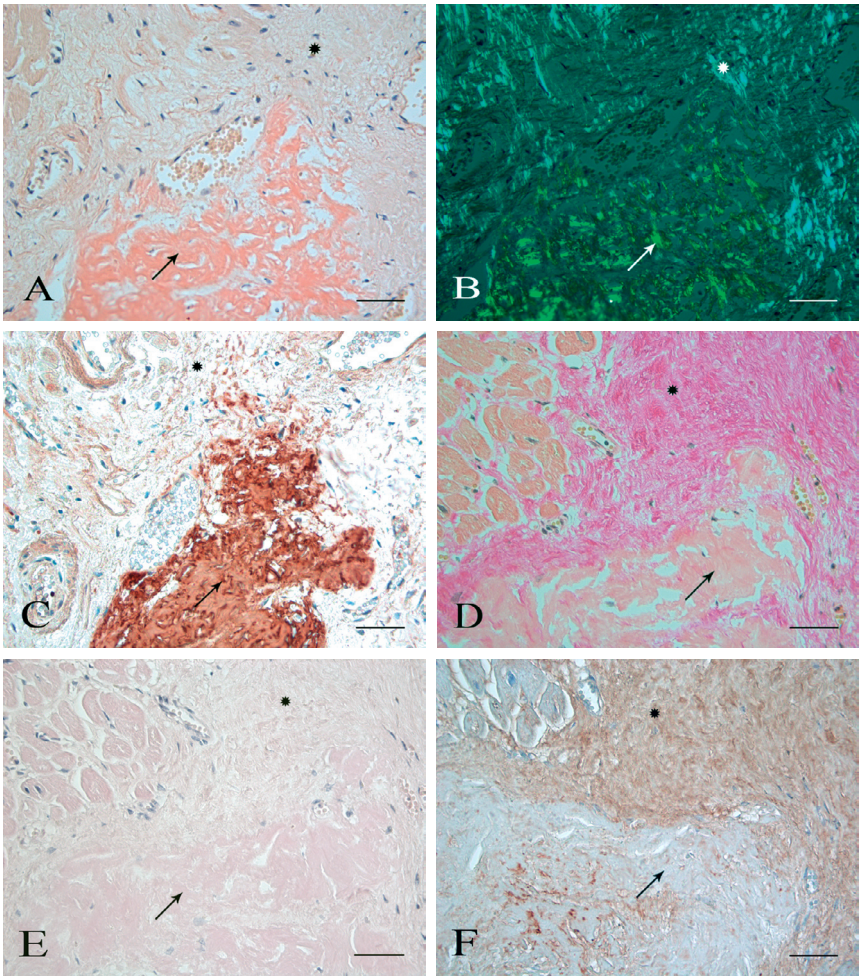

Figure 1.

Amyloid material deposited in the myocardium (arrow) in SSA shows red (A) to green (B) birefringence after Congo red staining (A,B) viewed in polarized light (B), and reactivity in anti-TTR immunohistochemistry (C). Fibrous scar tissue (asterisk), easy to recognize in the connective tissue staining (Herovichi; D) compared to HE staining (E), reacts in the anti-alpha2-macroglobulin immunohistochemistry (F). Original magnification: 400x. Scale bar: 100µm.

2.2. Senile systemic amyloidosis (SSA)

2.2.1. Definition, clinical characteristics, and histological findings

In SSA, wildtype TTR-derived amyloid (**Figure 1**) is deposited in parenchymal organs, mainly in the heart. Since amyloid in heart tissue was described 130 years ago (Soyka J. Prag Med Wschr 1: 165, 1876;. cited in Hodgkinson [Hodgkinson and Pomerance, 1977]), a substantial time elapsed until SSA

was precisely defined in 1984 (Pitkanen et al., 1984). The first step was the identification of prealbumin (TTR) in the amyloid deposits of SSA (referred to as "senile cardiac amyloidosis or "SCA" at that time; Sletten et al., 1980). As the exact composition of amyloid fibrils was not known at the time, a form of hereditary TTR-related amyloidosis called TTR Ile122Val (see Review of the literature, Chapter 2.2.4.2.), was first reported as "SSA" (Gorevic et al., 1989b; Jacobson et al., 1990). In 1995, the designation SSA was proposed "only for TTR amyloidosis occurring in advanced age and in the absence of TTR variant" (Gustavsson et al., 1995). The designation of SSA is included in the current nomenclature of amyloid diseases (**Table 2**; Westermark et al., 2005).

Previous results from hospital-derived series show that SSA is detected in one-fourth of individuals over 80 (Cornwell et al., 1983; Westermark et al., 2003), but population-based studies are lacking. SSA is clinically a benign disorder (Cornwell et al., 1995), although it can cause cardiac failure, conduction disorders, and arrhythmias (mainly atrial fibrillation observed in a few patients) (Johansson and Westermark, 1991; Pitkanen et al., 1984). Previously, a weak tendency for myocardial infarctions (MIs) in SSA patients has been proposed but no association with coronary atherosclerosis has been found (Cornwell et al., 1983). In an article by Johansson et al. (Johansson and Westermark, 1991), none of the twelve patients with massive amyloid infiltration due to SSA showed atrioventricular dissociation, and in none of the patients were conduction disturbances considered to represent the cause of death.

The diagnostic tools for cardiac amyloidosis include electrocardiography (ECG), echocardiography, angiocardiography, and technetium scanning (Stone, 1990; O'Hara and Falk, 2003). Recently, a promising noninvasive etiologic diagnosis of cardiac amyloidosis using ^{99m}Tc-3,3-Diphosphono-1,2-Propanodicarboxylic Acid Scintigraphy has been proposed (Perugini et al., 2005). However, for the purpose of identifying the insoluble amyloid fibrils in the tissue and defining the type of amyloid in order to choose a proper treatment, a histological sample is essential –(e.g. myocardial biopsy in the case of cardiac amyloidosis)– (Kyle et al., 1996; Hughes and McKenna, 2005). Even today, although it is possible to clinically diagnosis cardiac amyloidosis using the above mentioned diagnostic tools, SSA is mainly detected only by chance at the time of autopsy (Pitkanen et al., 1984; Kyle et al., 1996). Clinically, the AL (light chain, myeloma-associated) amyloidosis is the major differential diagnosis of SSA. The distinction is essential as SSA and AL amyloidosis are clearly distinct regarding prognosis and therapy (Kyle et al., 1996).Patients with AL amyloidosis benefit solely from chemotherapy, although they usually do not survive beyond six months (Dubrey et al., 1998; Dubrey et al., 2001). In contrast, the clinical consequences of SSA are generally minor, however recommendations for treating heart insufficiently are mostly not useful, and the use of digitalis preparations or excitative

β-adrenergic blockers may even be harmful (Kyle et al., 1996). Interestingly, a successful heart transplantation in a 68-year-old patient with SSA was recently reported (Fuchs et al., 2005).

In SSA, amyloid deposits have been detected in all tissues except the brain (Pitkanen et al., 1984). However, there are no reports on clinical consequences from SSA deposits in extracardial tissues. In most organs or tissues, amyloid deposits are mainly present in the blood vessel walls. However, in the heart they are also observed as diffuse or multifocal infiltrations between the heart muscle cells in the ventricles and atria (Cornwell et al., 1995), mostly sparing the specialized conduction tissue (Johansson and Westermark, 1991). Amyloid deposition in the myocardium leads to restrictive cardiomyopathy (RC). RC can be due to several conditions, and it can be divided into the primary and secondary types (Hughes and McKenna, 2005). The primary type of RC includes endomyocardial fibrosis, Löeffler's endocarditis, and idiopathic RC. The secondary type consists of non-infiltrative and infiltrative conditions. Non-infiltrative conditions include carcinoid heart disease and anthracyclin toxicity, while infiltrative conditions include amyloidosis, sarcoidosis, and the storage diseases. Determining the reason for RC is essential because the principles of medical treatment for amyloid cardiomyopathy clearly differ from other diseases causing RC (Olson et al., 1987; Hughes and McKenna, 2005).

2.2.2. Genetic and other risk factors for SSA

Apart from reports in non-population based series of age being a risk factor for SSA (Cornwell et al.; 1983, Westermark et al., 2003), no published data have been available on other risk factors.

2.2.3. Synthesis, structure, and function of transthyretin (TTR)

TTR, synthesized mainly in the liver (Skrede et al., 1975), is a 147 amino acid proprotein chain (MW 55kD), encoded by a single copy gene on chromosome 18. Some TTR synthesis also takes place in the choroid plexus in the brain (Li et al., 1997; Monteiro et al., 1998; Zheng et al., 1999), in the uvea (Kawaji et al., 2005), and in the islets of Langerhans (Jacobsson, 1989). After the signal sequence of 20 amino acids is cleaved off, the 127 amino acid monomeric TTR forms dimers and tetramers. The tetrameric form of TTR present in serum is capable of binding two molecules of thyroxine and has independent binding sites for retinal binding protein. Each monomer contains two segments of β-pleated sheets. Whereas the TTR variant associated with FAP invariably contains a mutation, the structure of the TTR protein in SSA is normal (Cornwell et al., 1988; Westermark et al., 1990b). In addition, no mutation in the gene coding for TTR has been detected (Christmanson et al., 1991; Gustavsson et al., 1995).

In the study by Gustavsson et al., the amyloid fibrils of SSA contained intact TTR together with a family of TTR fragments, the longest of which

consisted of the –COOH terminal amino acids 46-127 (Gustavsson et al., 1995). The proteolytic cleavage was not random, and the cleavage of the TTR molecule at the same positions has been described in TTR-related FAP as well (Gorevic et al., 1989c; Pras et al., 1983; Hermansen et al., 1995). Fragments of consistent length have been found in a number of SSA hearts, and in both FAP and SSA, these fragments may predominate over the presence of full-length TTR molecules (Cornwell et al., 1995). The mechanism of wildtype TTR-based amyloid fibril formation in humans is not understood completely, but the fragments are thought to have an important role (Cornwell et al., 1995). *In vitro* biophysical experiments indicate that the TTR fibrillogenesis process presumably requires the dissociation of the native tetramer into its constituent monomers (Kelly et al., 1997; Reixach et al., 2004). A conformational change within the monomer ("misfolding") enables the formation of soluble aggregates which become insoluble protofilaments. This may be followed by the self-assembly of four protofilaments to form amyloid fibrils (Kelly, 1998; Quintas et al., 2001; Reixach et al., 2004).

2.2.4. TTR-variants

More than one hundred variants of the TTR protein, arising from point mutations in its gene, have been discovered. These mutations destabilize the TTR molecule by lowering the energy requirement for tetrameric dissociation (Koo et al., 1999), and thus predisposing the molecule to fibril formation. Most of the variants are rare. The age of onset of the symptoms, the pattern of organ involvement, and the course of disease caused by the variants differ according to the type of mutation. The organs that are most often involved are the peripheral nerves (hence the name familial amyloidotic polyneuropathy; FAP) and the heart. Apart from the variants leading to clinical disease, there are also non-amyloidogenic variants. Only six TTR variants have been reported from Scandinavia (Suhr et al., 2003; Holmgren et al., 2005), and none in Finland. The nomenclature of the TTR variants in the current database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp) also takes the 20 amino acids signal sequence into account.

TTR Val30Met (replacement of methionine for valine at position 30) was the first variant described. It appears mainly in Portugal, Japan, Sweden, and the United States. The patients have systemic amyloidosis involving the autonomic nervous system and heart. Liver (Holmgren et al., 1991) and heart transplantation (Suhr et al., 2003) may stop the progress of the disease. The Val122Ile variant carried by 3.9% of African Americans and over 5% of the population in West Africa represents the most common amyloid-associated symptomatic TTR variant worldwide (Jacobson et al., 1990; Jacobson et al., 1997), with patients developing late-onset amyloid cardiomyopathy. Interestingly, this variant was originally described as "SSA"(Gorevic et al., 1989a). The variants Leu58His (Nichols et al., 1989) and Thr60Ala (Wallace

et al., 1986) cause carpal tunnel syndrome, with the latter also causing late-onset systemic amyloidosis with cardiac involvement and polyneuropathy (PNP). These variants are mainly found in the United States and Germany. The Leu55Pro variant of TTR is the most amyloidogenic variant (Yang et al., 2003) causing an early-onset aggressive diffuse amyloidosis with cardiac and neurologic involvement (Jacobson et al., 1992), and it has been described in the United States (original report) as well as Taiwan (Yamamoto et al., 1994). TTR Gly6Ser (designated as Gly26Ser in the present database: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp) is the most common TTR variant, with an allele frequency of 0.06-0.09 in Caucasians. The variant is not amyloidogenic but carriers have shown an increased affinity for thyroxine binding (Akbari et al., 1990), leading to euthyroid hyperthyroxinaemia.

2.3. Cerebral amyloid angiopathy (CAA)

2.3.1. Definition, clinical characteristics, and neuropathological findings

CAA is characterized by amyloid deposition in the cortical (**Figure 2A-C,F**) and leptomeningeal (**Figure 2E, F**) small- (**Figure 2A-C,E**) and medium-sized (**Figure 2F**) blood vessels, mainly in the arteries although veins and capillaries can also be affected (Vinters, 1987; Revesz et al., 2003). This phenomenon was originally described by Pantelakis (Pantelakis, p. 18) and thus became known as "congophilic angiopathy of Pantelakis". The most common form of CAA is the sporadic one, in which the major amyloid component consists of Aβ (Glenner and Wong, 1984a,b). Sporadic CAA is common in elderly individuals; its incidence in a general population aged ≥90 years was reported to range from 42.8% in men and 45.5% in women (Masuda et al., 1988) to 74% in both genders together (Masuda et al., 1988). The frequency of CAA escalates with increasing age (Masuda et al., 1988, Yamada et al., 1988 Xu et al., 2003). The role of CAA in normal aging is not clear, and only a few reports are available on animal models for sporadic CAA. Squirrel monkey (Saimiri spp.) is noted to develop significant CAA during natural aging, and this species is suggested to be a biologically advantageous model for studying the basic biology of idiopathic, age- related CAA (Elfenbein et al., 2007).

The distribution of CAA in the brain is patchy. This leads to difficulties in defining the extent of the occurrence and severity of the disease, as both depend on the number of brain areas included in the study. Due to this, several methods have been described in the literature to assess the extent and/or severity of CAA (**Table 5**).

In previous studies, parenchymal CAA was most frequently encountered

Table 5. Semi-quantitative methods used to estimate the severity of CAA in human post mortem brain sections.

Method/ Reference	Scale	Cerebral regions	Staining method	Individual score	Notes
Vonsattel et al. 1991	0-3	F, T, P, O	Congo red	-based on the most severe affected area only	-the extent of histological CAA changes has no effect on the severity of the disease -occipital lobe is not included
Olichney et al.1995	0-4	F, T, P, H	Thioflavin-S	-calculated by averaging the four regional scores	-the staining reaction may vanish with time
Ellis et al. 1996	0-3	F, T, P, H, E	Congo red* Thioflavin-S* Aβ IHC*	-calculated by averaging the five regional scores	-occipital lobe is not included - in some cases the diagnosis of amyloid is not based on recommended criteria
Thal et al. 2002	0-2	T (3 regions), O	Aβ IHC	-a simplified combination of the extent and local severity of histological changes	-determining an individual score may be impossible in some cases, e.g. mild CAA in several regions -the diagnosis of amyloid is not based on recommended criteria

F = frontal lobe; T = temporal lobe; P = parietal lobe; O = occipital lobe; H = hippocampus; E = entorhinal cortex.

*Note: Alternative methods; only one was used for each individual case.

in the frontal cortex, followed by the parietal and temporal lobes (Vinters and Gilbert, 1983; Ellis et al., 1996a). In AD patients, CAA has most often been encountered in the occipital cortex (Tian et al., 2003). The severity of CAA is associated with histopathologically determined AD changes: the neuritic plaques (Chapter 2.3.3.) and neurofibrillar tangles (Ellis et al., 1996; Yamada et al., 1988; Yamada, 2002). Clinically, CAA may be a diagnostic problem (Cederqvist et al., 1998). Mild CAA is not associated with clinical manifestations (Yamada, 2000), but severe CAA may cause cerebrovascular disorders such as cerebral hemorrhages and infarctions (Mandybur, 1986), leukoencephalopathy (Yamada, 2000), or dementia (Yoshimura et al., 1992; Greenberg et al., 1993; Olichney et al., 2000; Yamada et al., 1997a).

CAA-related hemorrhage commonly occurs in superficial lobar regions of the cerebrum (Yamada, 2000). This differs from typical hypertensive hemorrhages which typically are not cortical, but in contrast occur within deep locations of the brain, often near the basal ganglia. In addition, CAA-associated hemorrhages may occur in a normotensive patient, in contrast to the high blood pressure in patients with a hypertensive hemorrhage (Itoh et al., 1993). Further, CAA-related hemorrhage can be induced by anticoagulant

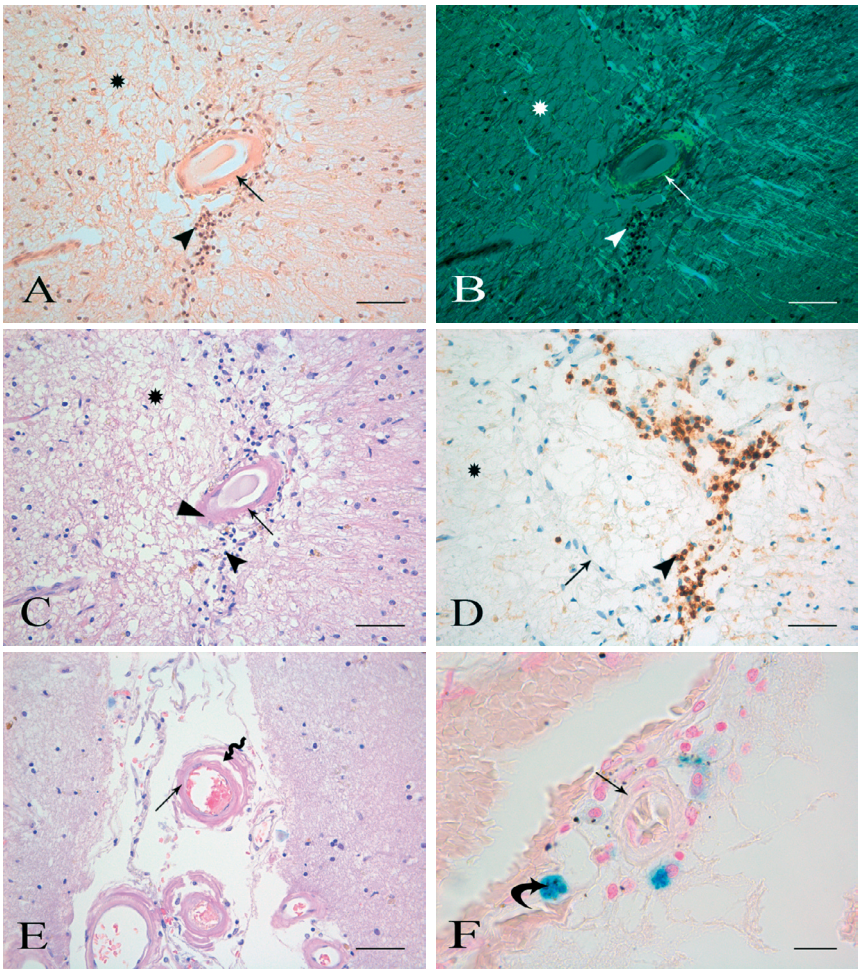

Figure 2.

A cerebral cortical blood vessel (arrow) is positive in the Congo red staining (A,B) in polarized light (B) and is surrounded by as small cortical infarction (asterisk; A-D) and associated with infiltration of leukocytes (arrow head; A-D), which react in the anti–common leukocyte antigen (CD45, D) immunohistochemistry. Other typical features of severe CAA include hyaline degeneration (triangle; C), double contour of the vessel wall or "double barrel" (curled arrow; E), and minor blood extravasation identified by finding iron in the cytoplasm of macrophages (bent arrow; F). C,E: HE staining. Original magnification: 400x (A-E); 1000x (F). Scale bar: 100µm (A-E); 25µm (F).

or antiplatelet therapy, and followed by a secondary subarachnoid hemorrhage (Itoh et al., 1993; Yamada et al., 1993). In addition to deposition of amyloid in the vascular walls (**Figure 2**) and subsequent obliteration of their lumens, histological features in severe CAA include the fibrinoid degeneration or necrosis of the vessel wall (**Figure 2C**; Okazaki et al., 1979, Rosenblum, 1977), the "double barrel lumen" (**Figure 2E**); Okazaki et al, 1979; Gilbert and Vinters, 1983), and microaneurysm formation (Okazaki et al., 1979;

Vinters and Roos, 1996).

2.3.2. Genetic and other risk factors for CAA

The Apo E locus is the major susceptibility gene for late-onset AD, as studied in a high-density whole-genome association study (Polvikoski et al., 1995a; Coon et al., 2007). The *APOE* ε4 allele is also strongly associated with increased vascular Aβ deposits (CAA) in AD (Schmechel et al., 1993; Premkumar et al., 1996). In contrast, the *APOE* ε2 allele is highly frequent in patients with CAA-related hemorrhage (McCarron and Nicoll, 1998). CAA has been demonstrated in 80-90% of AD patients (Esiri and Wilcock, 1986; Ellis et al., 1996; Yamada, 2000). In addition to being associated with AD (Ellis et al., 1996). CAA has also been associated with clinical dementia (Neuropathology Group. Medical Research Council Cognitive Function and Aging Study, 2001; Pfeifer et al., 2002).

The severity of CAA is associated with the presenilin (PS)-1 intronic polymorphism (Yamada et al., 1997b) and with the GT repeat polymorphism in the gene for neprilysin (Yamada et al., 2003). In AD patients, the severity of CAA has been associated with the α1-antichymotrypsin A allele (Yamada et al., 1998a), whereas the T/C polymorphism at codon 10 (exon 1) of the TGFβ gene has recently been noted to be associated with the severity of CAA especially in non-AD patients and non -APOE ε4 carriers (Hamaguchi et al., 2005). Some polymorphisms that have been shown to not be associated with CAA in humans include the: butyrylcholinesterase-K variant (Yamada et al., 1998b), exon 18 polymorphism of the α2M gene (Yamada et al., 1999b), interleukin-1A allele 2 (McCarron et al., 2003), cathepsin D exon 2 (C to T) polymorphism (Davidson et al., 2006), and in another study, the α1-antichymotrypsin polymorphism (Sodeyama et al., 1999).

The increased plasma homocystein levels are not associated with CAA (Irizarry et al., 2005). The development of CAA does not correlate with the presence of common cerebrovascular risk factors such as hypertension, diabetes mellitus, and hyperlipidemia (Yamada, 2000). The role of arteriosclerosis in CAA is somewhat unclear. A Japanese study did not detect a correlation between CAA and severity of atherosclerosis in the cerebral arteries (Yamada et al., 1987), although in a North American study a positive correlation between CAA and cerebral arteriosclerosis was found (Ellis et al., 1996).

2.3.3. Synthesis and structure of amyloid beta protein (Aβ)

In 1984, Glenner and Wong reported the isolation of a 4200-dalton polypeptide, β-protein or Aβ, from amyloidotic vessels of AD and Down syndrome patients (Glenner and Wong, 1984a,b). This polypeptide was not present in any of the age-matched normal cerebral vessels they examined. By immunizing mice against a synthetic peptide identical to the first 10 residues of the β-protein and then using the antiserum immunohistochemically to

stain amyloid in cerebral vessels, this protein was detected in neuritic plaques one year later (Masters et al., 1985). The term "neuritic plaque" refers to plaques with thickened silver-positive neuritis (Mirra and Heyman, 1991) in which Aβ is aggregated into fibrils and forms amyloid (Yamaguchi et al., 1990; Dickson, 1997). In contrast, plaques referred to as "diffuse" are devoid of abnormal neuritis and fibrillar amyloid. By using immunohistochemical techniques, several other types of plaques have later been identified (Delaere et al., 1991). In CAA, amyloid is formed from aggregated Aβ, similar to the neuritic plaques.

Aβ is a proteolytic fragment of the membrane-spanning amyloid precursor protein APP (Kang et al., 1987). *In vivo*, soluble Aβ is thought to gradually transform into the fibrillar form (Selkoe, 1991). Aβ has several isoforms, which tend to appear in varying localizations within various brain regions (Vinters et al., 1996). Biochemical analyses have indicated chemical distinctions in parenchymal and vascular amyloid (Prelli et al., 1988). For example, the carboxy-terminus of cerebrovascular Aβ is three amino acid residues shorter than Aβ in senile plaques (39 vs. 42 residues). In addition, the Aβ species deposited in blood vessel walls show both C- and N-terminal heterogeneity which can be demonstrated with specific antibodies (Revesz et al., 2003). Both immunohistochemical and biochemical studies have demonstrated that Aβ species ending at position 40 are predominantly deposited in the vessel walls in both sporadic and familial AD as well as in HCHWA-D (Castano et al., 1996). On the other hand, in addition to being present, Aβ 42 is sometimes the sole deposited peptide species in CAA in both humans and transgenic animals (Van Dorpe et al., 2000). These observations, also supported by *in vitro* experiments (Lansbury, 1997), suggest that the first Aβ species that are deposited in the vessel wall end at position 42, while the more soluble Aβ 40 is subsequently entrapped (Van Dorpe et al., 2000). An alternative possibility is that Aβ 40 is produced *in situ* by degradation of Aβ 42 by carboxypeptidases (Revesz et al., 2003). The N-terminal heterogeneity of Aβ, with a potential for enhanced aggregation, is also documented in CAA (Tekirian et al., 1998). This may imply that tissue-specific endopeptidases may determine the differential processing of APP in the brain parenchyma and microvessel wall (Vinters et al., 1996).

The origin of both vascular and plaque Aβ in the brain is under debate. Vascular Aβ has been suggested to originate from blood or CSF, or as a local product of the smooth muscle cells in the blood vessel walls (Wisniewski and Wegiel, 1994; Vinters et al., 1996). Senile plaques are found in close proximity to brain capillaries (Miyakawa and Uehara, 1979; Miyakawa et al., 1982). This lead to the hypothesis that capillary degeneration with resulting amyloid fibril formation might be a primary alteration in the genesis of senile plaques, yet another study suggests that the role of capillary degeneration in the evolution of senile plaques is minor (Kawai et al., 1990). Several types of structural alterations associated with amyloid deposition have been noted

in dendrites such as loss of dendritic spines, shaft atrophy, bending, abrupt branch endings, varicosity formation, and sprouting, in both a transgenic mouse model of AD and in human postmortem brain (Grutzendler et al., 2007). These alterations may be due to the potential cytotoxicity of Aβ.

The production of Aβ is under debate. In the classical view, the precursor protein AβPP is cleaved at the plasma membrane by α-, β-, and δ- secretases to produce Aβ, and that the cleavage product Aβ is rapidly expelled from the cell thereafter. Fitting this idea, the Aβ deposits in AD have traditionally been considered extracellular in nature. In addition to the cell surface, AβPP has been detected inside the cell in the endoplasmic reticulum and Golgi apparatus, and in recent studies using fluorescence resonance energy transfer and confocal microscopy, in human neuroglioma cells in close association to the centrosome (Nizzari et al., 2007). However, growing evidence indicates that Aβ peptide itself may accumulate intraneuronally. After the first observation in human neurons (Grundke-Iqbal et al., 1989), several reports on intracellular Aβ in human diseases and animal models have been presented (Chen et al., 2006; LaFerla et al., 2007), suggesting that Aβ can also be generated intracellularly (Wertkin et al., 1993; Tienari et al., 1996; Hartmann et al., 1997).

Apart from neural tissue, Aβ is also detectable in the plasma and CSF in concentrations that hold the pool of brain Aβ in equilibrium with them. The influx of soluble Aβ across the blood brain barrier (BBB) depends on its interaction with the receptor for advanced glycocylated end products (RAGE) and low-density lipoprotein receptor (LRP) in the endothelium (Deane et al., 2004; Zlokovic, 2005). The clearance of Aβ from the brain and its implications in AD and CAA are discussed in detail in the Discussion (Chapter 6.4).

Although Aβ expression is mainly restricted to the brain, its precursor protein AβPP is ubiquitously expressed in mammalian cells with a broad tissue distribution (Fardilha et al., 2007). In addition to cerebral cortical neurons, AβPP has also been detected in other neural cells (neurons and satellite glial cells of the dorsal root, enteric and trigeminal ganglia, and the adeno- and neurohypophysis), as well as in extraneuronal cells (megakaryocytes and adrenal gland cells) in samples from both AD patients and non-AD individuals (Selkoe et al, 1988; Arai et al., 1991). More recently, AβPP has also been reported in epidermal melanocytes and melanoma cells (Quast et al., 2003), thyroid cells and keratinocytes (Pietrzik et al., 1998; Schmitz et al., 2002), human sperm (Fardilha et al., 2007), malignant tumors such as oral squamous cell carcinoma (Lin et al., 2002), and neuronal (Nakagawa et al., 1999) and gastrointestinal (Meng et al., 2001; Hansel et al., 2003) carcinoma cells.

2.3.4. Alzheimer's disease (AD), Aβ, and neuroinflammation

The previously mentioned extracellular accumulation of Aβ as amyloid

plaques is one of the histopathological hallmarks of AD. During the last 15 years, numerous inflammatory mediators have been observed (Akiyama et al., 2000), including complement proteins (Eikelenboom and Veerhuis, 1996). Only after it was accepted that the brain was not an immunologically isolated organ (Hickey and Kimura, 1988), has increasing information based on animal models and clinical studies accumulated about the involvement of inflammation in AD pathogenesis. The chronic inflammatory response in AD is closely associated with amyloid plaques (Rogers et al., 2002), and locally induced by Aβ without any apparent influx of leukocytes from the circulation (Rogers et al., 1992; Eikelenboom et al., 2000). A long list of inflammatory mediators such as complement proteins, cyto- and chemokines, cyclooxygenases, coagulatory and fibrinolytic proteins, β-integrins, and free radicals have been encountered in AD (Eikelenboom and Stam, 1982; Akiyama et al., 2000). However, at present none of these factors, synthesized by neurons, astrocytes, and microglia (Bergamaschini et al., 2001; Veerhuis et al., 1999), is considered to be a principal factor in AD pathophysiology (Akiyama et al., 2000).

In *in vitro* studies, Aβ peptide induces an inflammatory cascade with the expression of cytokines and CD40 in human vascular cells (Suo et al., 1998), in close proximity to amyloid plaques (Rogers et al., 2002). Further, in familial British dementia (FBD) and familial Danish dementia (FDD), deposition of the cerebrovascular amyloid proteins ABri and ADan, respectively, associates with complement activation using both the classical and alternate pathways (Rostagno et al., 2002) (Ghiso et al., 2000; Holton et al., 2002). These forms of dementia carry striking neuropathological similarities with AD, in particular with CAA (Rostagno et al., 2002).

Complement proteins are commonly encountered in parenchymal Aβ deposits (senile plaques), including C3d (McGeer et al., 1989) and membrane attack complex (Akiyama et al., 2000). Aggregated Aβ is able to activate complement in an antibody-independent fashion, by binding to C1q (Yasojima et al., 1999; Rogers et al., 2002). Complement is also activated in human brain tissue in association with infarction (Lindsberg et al., 1996) and immunological neurological disease (Sanders et al., 1986; Compston et al., 1989), as well as in the cerebrospinal fluid following subarachnoid hemorrhage (Lindsberg et al., 1996).

2.3.5. Origin of CAA

The origin of the amyloid proteins, especially fibril protein Aβ, deposited in the cerebral blood vessels, is poorly understood. Different theories about the origin of CAA (**Table 6**) have been proposed (Revesz et al., 2003).

According to the systemic theory, the cerebrovascular Aβ deposits are derived from the circulation, like the amyloid deposits in systemic amyloidosis.

This is supported by the observation that AβPP is expressed by most cell types in the body, and that AβPP is detectable in plasma (Zlokovic, 2002).

Table 6. Theories on the origin of CAA.

Name of the theory	Source of Aβ	Mediated by	Arguments against*
Systemic[a]	a) Circulation, across the BBB b) CSF	-several receptors at BBB: e.g. RAGE at the luminal side and LRP-1 at the abluminal (brain) side	-Aβ is first detectable at the abluminal (brain) side -AβPP transgenic mice show no CNS changes at a very advanced age
Vascular[b]	Local production by cerebrovascular cellular elements, e.g. smooth muscle cells		-Aβ deposits have been detected in capillaries -larger arteries are less affected than the smaller vessels
Drainage[c]	Neurons in the CNS	-draining and concomitant deposition of Aβ along the periarterial perivascular spaces	

CAA = cerebral amyloid angiopathy; Aβ = amyloid β-protein; BBB = brain blood barrier; CSF = cerebrospinal fluid;
RAGE = receptor for advanced glycation end-products; AβPP = amyloid β precursor protein; CNS = central nervous system; LRP = low-density lipoprotein receptor-related protein.

*See text: Review of the Literature, Chapter 2.3.5.

[a]Zlocovic, 2002.

[b]Frackowiak et al., 1994.

[c]Weller et al., 1998.

The two-directional receptor-mediated transport of Aβ across the BBB and its exchange between the CNS, blood, and CNF, in part determines the concentration of Aβ in the CNS. Several receptors have been implied in this process such as RAGE, LRP-1, scavenger receptor, and the megalin receptor (Zlokovic, 2002). RAGE-mediated transport of Aβ takes place at the luminal side of the BBB, facilitating Aβ transport in the luminal to abluminal direction, and allowing a significant influx of Aβ into the brain (Zlokovic, 2002). In contrast, LRP-1 receptor-mediated transport is initiated abluminally (i.e. CNS side) and thus has a role in eliminating Aβ from the cerebral interstitial fluids (Zlokovic, 2002). One of the factors modulating this process is APOE, which is a ligand of LRP-1. APOE is a confirmed risk factor for AD (Polvikoski et al., 1995), but interestingly, it is also a risk factor for CAA. The arguments against a blood-borne source of cerebrovascular Aβ is firstly that morphological Aβ is initially detectable in the abluminal (brain side) basement membrane of blood vessels (Yamaguchi et al., 1992). Secondly, trangenic mice overexpressing the 99-amino-acid C-terminal region of the human AβPP in multiple tissues, including exceptionally high levels in the plasma, showed no CNS changes up to age 29 months (Fukuchi et al., 1996).

Another source of Aβ in CAA could be the CSF, since Aβ is found in the CSF in both individuals with normal cognition and in AD patients (Ida et al., 1996; Ghiso et al., 1997). An argument supporting this hypothesis of origin is that in CAA small arteries are more commonly affected than the larger blood vessels in the subarachnoid space (Revesz et al., 2003).

The vascular hypothesis suggests that Aβ is derived from cerebrovascular cellular elements. Morphologically, Aβ has been shown to be closely associated with cerebrovascular smooth muscle cells (Frackowiak et al., 1994). Isolated cerebral microvessels and meningeal blood vessels are able to produce Aβ (Kalaria et al., 1996). Aβ is also expressed by smooth muscle cells, pericytes, and endothelial cells (Burgermeister et al., 2000). Some arguments against the vascular hypothesis are that Aβ deposits have also been detected in capillaries, and that larger arteries with several layers of smooth muscle cells are less affected than smaller vessels (Weller et al., 1998).

The drainage hypothesis proposes that Aβ, produced primarily by CNS neurons, is drained along the periarterial perivascular spaces of the brain parenchyma and leptomeninges, and that CAA occurs due to Aβ deposition along these drainage pathway vessels (Weller et al., 1998). Degenerative vascular changes, which commonly affect aged individuals, could have a deleterious effect on the perivascular flow of interstitial fluid and be a factor in the vascular deposition of Aβ. The presence of CAA in transgenic animal models of AD, in which AβPP is expressed by neurons, could also support the drainage hypothesis (Burgermeister et al., 2000; Van Dorpe et al., 2000).

Some of the amyloid peptides implicated in familial CAAs, such as ACys, ATTR, and AGel, deposit in systemic organs, supporting the systemic theory. In both FBD and FDD, the origin of the amyloid plaques is not known. BriPP mRNA is expressed in normal human brain (Vidal et al., 1999), indicating possible local production. On the other hand, ABri is also found in the circulation and in the tissues (Ghiso et al., 2001) and thus may also be peripherally produced (Revesz et al., 2003).

2.3.6. Hereditary forms of CAA

The familial forms of CAA are rare. The most important hereditary condition associated with CAA is hereditary AD, which is caused by mutations in several genes. Hereditary AD is inherited in an autosomal dominant manner with almost complete gene penetrance in only about 5% of all cases (Revesz et al., 2003).

In the gene for *AβPP*, which is present on chromosome 21, several missense mutations have been detected. These mutations are located within or just outside the region encoding the Aβ peptide, near one of the secretase cleaving sites. These mutations exert their pathogenic effect through different mechanisms and often have characteristic clinical and pathological phenotypes. Severe CAA can also be associated with AD in cases where there are mutations in the *PS-1* and *PS-2* genes, or in the gene for *cystatin-C*,

with the latter leading to HCHWA-I. Leptomeninges and meningeal vessels can also be affected by familial TTR amyloidosis (Benson, 1996), especially prominent in the Hungarian (D18G) and Ohio (V30G) mutations (Garzuly et al., 1996; Petersen et al., 1997; Vidal et al., 1996). In AGel (Kiuru, 1998a) and prion protein amyloidosis (Ghetti et al., 1996), CAA has also been described. In familial British (FBD) and Danish (FBD) dementia, severe CAA is one of the pathological hallmarks. In these diseases, the genetic abnormality results in elongation of the 266-amino-acid wildtype precursor protein designated as BRI-PP. This precursor protein is processed, resulting in the secretion of mutated C-terminal peptides that are 34 amino acids in length (Kim et al., 1999). The amyloid peptides designated as ABri in FBD, and ADan in FDD, have unique 12-amino-acid C-terminal sequences that have allowed for the generation of "mutation-specific" antibodies, which exclusively recognize either ABri or ADan (Revesz et al., 2003).

2.3.7. Tau protein and neurodegeneration

The other pathogenomic lesion of AD, described already by Alois Alzheimer and the main constituent of the intraneuronal neurofibrillar tangles and extraneuronal neurofibrillary threads, is the microtubule-associated protein tau (Grundke-Iqubal et al., 1986a). These filamentous structures have also been encountered in other neurodegenerative diseases such as progressive supranuclear palsy, corticobasal degeneration, Pick's disease, argyrophilic grain disease, and inherited frontotemporal dementia and parkinsonism linked to chromosome 17 (Wilhelmsen et al., 1994; Baker et al., 1999; Fallin, 2001; Goedert, 2005). In intracellular neuronal deposits, tau protein is mainly in the phosphorylated, toxic form (Grundke-Iqubal et al., 1986b) as paired helical filaments. In adult human brain, tau exists in six major isoforms, which are generated by alternative splicing of exons 2, 3 and 10 of the *tau* gene (Goedert, 2005). In AD, the neurofibrillar tangles contain all six major tau isoforms. In contrast, in for example progressive supranuclear palsy, only 4-repeat tau is found. Phosphorylated tau also accumulates in the inclusion bodies of diseased muscle cells in sporadic inclusion body myositis (Askanas et al., 1994). In addition, tau epitopes have been detected in oculopharyngeal and Becker muscular dystrophy, dermatomyositis, central core disease, neurogenic atrophy, and in the recovery phase of an attack of malignant hyperthermia (Lubke et., 1994). In animals, tau is widely expressed and can be found in the heart, skleletal muscle, lung, kidney, testis, adrenal gland, stomach, and liver (Ashman et al., 1992; Gu et al., 1996).

2.4. Hereditary gelsolin (AGel) amyloidosis

2.4.1. Definition, clinical characteristics, and histopathological findings

Hereditary gelsolin (AGel) amyloidosis is an autosomal dominant form of systemic amyloidosis originally reported from Finland (Meretoja, 1969), and thereafter from several European countries, North America and Japan (Conceicao et al., 2003; Kiuru, 1998; Makishita et al., 1996; Stewart et al., 2000). The most prominent clinical features, after onset in the thirties, are slowly progressive corneal lattice dystrophy, cranial neuropathy, and cutis laxa. AGel amyloidosis is caused by a G654A, or rarely, G654T mutation in the gene coding for gelsolin on chromosome 9 (Levy et al., 1990; de la Chapelle et al., 1992). Neurological involvement in AGel amyloidosis ranges from cranial and peripheral to autonomic nerves and the central nervous system (Meretoja, 1969; Kiuru et al., 1994; Kiuru et al., 1999; Rosenberg et al., 2001). The most common neurological feature is slowly progressive cranial neuropathy with onset in the forties, and predominant facial and lower cranial nerve involvement that can cause severe bulbar paresis at old age. Some patients who were originally published as atypical bulbar amyotrophic lateral sclerosis (Klaus et al., 1959) were later shown to carry the G654T gelsolin gene defect (de la Chapelle et al., 1992). Autonomic and cerebral involvement with AGel deposition is usually less significant (Akiya et al., 1996; Kiuru, 1998). Although the disease may cause ataxia of gait and minor (mainly sensory) somatic nerve involvement (Kiuru, 1998), it has not previously been shown to be lethal.

2.4.2. Synthesis, structure, and function of gelsolin

Gelsolin is the principal actin-severing protein in mammalian leukocytes, platelets, and other cells with powerful effects on actin (Janmey et al., 1985; Kwiatkowski and Yin, 1987). In addition to the cytoplasmic form, gelsolin also appears in the plasma wherein the majority is derived from muscle (Kwiatkowski et al., 1988). Both forms of gelsolin are encoded by a single gene (Kwiatkowski and Yin, 1987). *In vitro* studies have shown that the G654A and G654T gelsolin gene mutations do not interfere with the normal actin-modulating function of intracellular gelsolin. Moreover, the aberrant processing of secreted mutant gelsolin to AGel amyloid precursor takes place in a gelsolin-negative background, suggesting that the symptoms are caused by the accumulation of amyloid derived from secreted plasma gelsolin in the tissues (Kangas et al., 1999) rather than the functional aberration of cytoplasmic gelsolin. In specific tissues, the larger precursor protein is cleaved first by furin, a proprotein convertase (Chen et al., 2001), and thereafter by a MT1-MMP-like protease (Page et al., 2005) to yield the amyloidogenic peptides.

2.5. Medin (AMed) amyloidosis

2.5.1. Definition and histopathological findings

Aortic medial or medin (AMed) amyloidosis is a very common local form of an old age-associated amyloid disorder (Schwarz P. New patho-anatomic observations on amyloid in the aged. Fluorescence microscopic investigations. In: Mandema E, Ruinen L, Scholten JH, Cohen AS, editors. Amyloidosis. Amsterdam: Excerpta Medica, 1968. pp. 400-4155; Battaglia and Trentini, 1978; Cornwell et al., 1982; Cornwell et al., 1983). It occurs in virtually all individuals aged ≥ 60 years (Haggqvist et al., 1999; Reches and Gazit, 2004). AMed amyloid is also found in other arteries, mainly in the upper part of the body, including the temporal and basilar arteries (Peng et al., 2002; Peng et al., 2005). In AMed amyloidosis, the individual amyloid deposits are small and situated in close association to the internal elastic lamina (Peng et al., 2005). Interestingly, in giant cell arteritis of temporal arteries, AMed amyloid is found within the inflammatory exudate in the blood vessel wall (Peng et al., 2002).

2.5.2. Synthesis, structure, and function of lactadherin

Medin, a 50-amino-acid internal fragment of the 364-amino-acid precursor protein lactadherin, also called milk fat globule protein or BA46 (Couto et al., 1996, Andersen et al., 2000), is the main amyloid constituent of aortic medial amyloid. The amyloid fibril protein, called medin, corresponds to positions 245-294 of lactadherin (Haggqvist et al., 1999). The functions of lactadherin are poorly understood. Lactadherin is expressed by breast epithelial cells and thus occurs in milk. It has also been proposed to provide protection against rotavirus infection (Newburg et al., 1998) and is suggested to have properties important for the coagulation system (Peng et al., 2002). Apart from breast epithelial cells, lactadherin is expressed in several kinds of cells including smooth muscle cells (Peng et al., 2005). Thus, medin synthesis is suggested to be local, supported by the finding that although both water-soluble and -insoluble lactadherin is found when analyzing proteins extracted from amyloid-rich aortic media, only insoluble medin could be detected in the amyloid fraction (Peng et al., 2005).

3. THERAPY

After introduction of the term "amyloid" into medical literature, more than a century elapsed until any therapy was available for amyloid diseases. The first steps were taken in AA (inflammation-associated) amyloidosis in which it was noted that when the infective focus was removed, the development

of amyloid accumulation could be hindered (Waldenström, 1976). In 1986, Zemer showed that in familial Mediterranean fever colchicine is able to, apart from preventing attacks of the disease, hinder the development of AA amyloidosis which is a late complication of the disease (Zemer et al., 1986). An evaluation of the effects of amyloid disease therapy has been possible since 1988 when Hawkins and Pepys introduced 123I-SAP (serum amyloid P–component) scintigraphy as a clinical method to visualize the presence of amyloid in organs (Hawkins et al., 1988). In 1991, Holmgren introduced liver transplantation as a treatment for hereditary TTR amyloidosis (Holmgren et al., 1991). Since 1996, remarkable advances in medical therapies for AL amyloidosis have also taken place (Skinner et al., 1996).

Several compounds have been investigated for their usefulness as medical therapies for amyloid diseases. Logical targets for therapy would focus on common amyloid features such as SAP binding, the presence of the glycosaminoglycan perlecan, and the fibrillar structure of amyloid (Buxbaum, 2004). Alternative therapeutic targets could utilize specific features of individual amyloid proteins.

SAP, a protein associated with all amyloids, is able to inhibit proteolysis of amyloid fibrils *in vitro*, and slows AA amyloid deposition in mice devoid of SAP (Botto et al., 1997). In AL amyloidosis, a specific inhibitor of SAP binding that reduces SAP levels has been administered in a phase I/II study. This compound was quite safe in the initial report (Pepys et al., 2002), but its efficacy and safety in humans is not yet known (Buxbaum, 2004). Anionic sulfonates, such as glycoaminoglycans, may also be used in order to prevent amyloid formation (Kisilevsky et al., 1995), and they have been shown to be active in the treatment of murine inflammation-induced AA amyloidosis (Kisilevsky and Szarek, 2002; Kisilevsky et al., 2007). Transgenic mice carrying a mutant human Aβ protein precursor develop amyloid plaques in their brains, analogous to those seen in human AD. Promising experiments actively or passively immunizing these mice against the Aβ 1-42 peptide, resulted in shrinking of the plaques in the CNS (Janus et al., 2000; Schenk et al., 1999). A vaccine trial with the same immunogen was initiated in human patients with advanced AD, but was stopped because some of the participants developed evidence of severe CNS inflammation (Nicoll et al., 2003). An immunogenic approach targeting AL has also been investigated in mice (Hrncic et al., 2000), and animal models of immunization against selected TTR mutants have recently been reported (Terazaki et al., 2006).

In vitro, Aβ fibril formation has been inhibited by preventing the β sheet conformation by peptides that bind to Aβ (Soto et al., 1996). *In vitro* studies have also demonstrated that several nonsteroidal anti-inflammatory drugs and structurally similar compounds are able to inhibit TTR-amyloid fibril formation, based on these drugs complexing with TTR which leads to the stabilization of the tetrameric conformation of TTR. In addition, several

small molecules that inhibit TTR fibril formation have been identified or synthetized (Klabunde et al., 2000; Hammarstrom et al., 2003). Some of them have even been tested in transgenic mouse models (Buxbaum, 2004). These molecules include genistein and chromium (III) ion. Genistein, the major isoflavone natural product of soy, has been reported to be safe for human consumption and to reduce *in vitro* fibril formation of wildtype TTR, and the Val50Met and Val122Ile TTR variants (Green et al., 2005). The chromium (III) ion increases the *in vitro* binding capacity of thyroxin (T4) to the wildtype and Val30Met variant of TTR, thus suppressing amyloid fibril formation (Sato et al., 2006).

AIMS OF THE STUDY

The aims of the present study were to:

1. Investigate the frequency of SSA and CAA in an elderly Finnish population (I, II, III)
2. Investigate health-associated and genetic risk factors for SSA and CAA (I, II, III)
3. Study the possible association between SSA and CAA (III)
4. Identify possible new amyloid-associated proteins in SSA, CAA, and AGel amyloidosis (I, II, IV)

MATERIALS AND METHODS:

1. SUBJECTS

1.1. Vantaa 85+ study protocol and participants (I, II, III)

The Vantaa 85+ study included all persons aged 85 years or more and living in the city of Vantaa (Southern Finland) on April 1, 1991. Of the 601 eligible subjects, 553 participated in the study, while most of the remaining individuals died before they could be examined. The clinical follow-up studies were carried out in 1994, 1996, 1999, 2001, and, 2003. The present study is based on the results of consented post mortem examinations, performed in 51% of the Vantaa 85+ study material. This autopsy rate is worldwide the second highest among all prospective population-based studies with neuropathological examination of the subjects (Zaccai et al., 2006). Thus far, the study material has yielded several publications, the most notable of which are presented in **Table 7**.

1.1.1. SSA study (I)

The SSA study (I) included 256 subjects ≥ 85 years of age, ranging from 85.1-105.6 years (mean 92.5 years), from whom both histological myocardial specimens and blood samples were available. 84% (214/256) of the subjects were females and 16% (42/256) were males.

1.1.2. CAA study (II)

The CAA study (II) was based on post mortem specimens from 74 individuals ≥ 95 years old, with ages ranging from 95.0-105.6 years (mean 96.6), including all individuals in whom the required specimens from five brain areas for CAA diagnostics were available. 82% (61/74) were females with ranging from 95.1-105.6 years (mean 96.9), while 18% (13/74) were males ranging from 95.0-98.7 years (mean 95.6).

1.1.3. Association between SSA and CAA (III)

Association between SSA and CAA was studied on all 63 individuals, in whom both the myocardial and cerebral cortical specimens were available. The age range of this population was 95-105.6 years (mean 98). 84% (53/63) of the subjects were females whereas 16% (10/63) were males.

Table 7. A list of selected publications on the Vantaa 85+ Study material.

Authors	Title	Publication
Genetic risk factor studies		
Enattah et al.	Genetic variant of lactase-persistent C/T-13910 is associated with bone fractures in very old age	J Am Geriatr Soc 2005
Myllykangas et al.	Chromosome 21 BACE2 haplotype associates with Alzheimer's disease: a two-stage study.	J Neurol Sci 2005
Lambert et al.	Contribution of APOE promoter polymorphisms to Alzheimer's disease risk.	Neurology 2002
Myllykangas et al.	ApoE epsilon3-haplotype modulates Alzheimer beta-amyloid deposition in the brain.	Am J Med Genet 2002
Myllykangas et al.	Association of lipoprotein lipase Ser447Ter polymorphism with brain infarction: a population-based neuropathological study.	Ann Med 2001
Baker et al.	No association between TAU haplotype and Alzheimer's disease in population or clinic based series or in familial disease.	Neurosci Lett 2000
Juva et al.	Apolipoprotein E, cognitive function, and dementia in a general population aged 85 years and over.	Int Psychogeriatr 2000
Myllykangas et al.	Cardiovascular risk factors and Alzheimer's disease: a genetic association study in a population aged 85 or over.	Neurosci Lett 2000
Myllykangas et al.	Genetic association of alpha2-macroglobulin with Alzheimer's disease in a Finnish elderly population.	Ann Neurol 1999
Sulkava et al.	APOE alleles in Alzheimer's disease and vascular dementia in a population aged 85+.	Neurobiol Aging 1996
Polvikoski et al.	Apolipoprotein E, dementia, and cortical deposition of beta-amyloid protein.	N Engl J Med 1995
Other epidemiological studies		
Barkhof et al.	The significance of medial temporal lobe atrophy: a postmortem MRI study in the very old.	Neurology 2007
Rastas et al.	Atrial fibrillation, stroke, and cognition: a longitudinal population-based study of people aged 85 and older.	Stroke 2007
Polvikoski et al.	Incidence of dementia in very elderly individuals: a clinical, neuropathological and molecular genetic study.	Neuroepidemiol 2006
Rastas et al.	Association between blood pressure and survival over 9 years in a general population aged 85 and older.	J Am Geriatr Soc 2006
Rastas et al.	Association of apolipoprotein E genotypes, blood pressure, blood lipids and ECG abnormalities in a general population aged 85+.	BMC Geriatr 2004
Polvikoski et al.	Prevalence of Alzheimer's disease in very elderly people: a prospective neuropathological study.	Neurology 2001
Rahkonen et al.	Delirium in the non-demented oldest old in the general population: risk factors and prognosis.	Int J Geriatr Psychiatry 2001
Juva et al.	APOE epsilon4 does not predict mortality, cognitive decline, or dementia in the oldest old.	Neurology 2000
Päivärinta et al.	The prevalence and associates of depressive disorders in the oldest-old Finns.	Soc Psychiatry Epidemiol 1999
Kiljunen et al.	Depression measured by the Zung Depression Status Inventory is very rare in a Finnish population aged 85 years and over.	Int Psychogeriatr 1997

1.1.4. Blood samples (I, II)

The frozen blood samples for DNA testing were available from 86% of the entire study population (517/601). All 256 participants in study I, and all 63 participants in study III, were genetically tested. In study II, a blood sample was available from 96% (71/74) of the neuropathologically studied individuals.

1.2. Case report on AGel amyloidosis (IV)

The report on advanced Gelsolin amyloidosis describes a 78-year old male referred to the Department of Neurology at Helsinki University Central Hospital in Helsinki, Finland for the evaluation of progressive imbalance. After one year, the patient became bedridden, had respiratory and subsequent gastrointestinal infections, and died at the age of 79.

1.3. Medin study

The occurrence of medin in CAA was studied in histological cerebral cortical samples from ten individuals with clinical AD and seven controls with normal cognition.

2. CLINICAL AND POST MORTEM INVESTIGATION

2.1. Vantaa 85+ study (I, II, III)

2.1.1. Interview and clinical examination of the subjects (I, II, III)

The Vantaa 85+ study protocol was based on interviews of the subjects on several medical, social, economical and health-associated parameters, as well as clinical examinations including a thorough neurological study (Polvikoski et al., 1995).

The clinical data was obtained in the baseline study in 1992 and several follow-ups (Myllykangas et al., 2001). In this study, several health-associated factors were included. Subjects were classified as having diabetes mellitus (DM; I) if they used blood glucose lowering medication or insulin, and hypetension (HT; I) if they used any blood pressure lowering medication. The BMI values (body mass index, weight divided by height squared; I) were classified into five categories: $1 \geq 32$; 2 = 28-31; 3 = 19-27; 4 = 15-18; $5 \leq 14$.

If an exact measurement was not possible, the BMI category was estimated. The clinical diagnosis of dementia (II, III) was performed according to the guidelines of the American Psychiatric Association (1987) and included memory, behavior, emotional, and social function tests. The clinical diagnosis of AD (II, III) was based on the NINCDS-ADRDA criteria (McKhann et al., 1984), which is mainly based on the exclusion of other clinically significant causes of dementia, e.g. cerebrovascular disease or Parkinsonism. In the present study, the clinical diagnosis of dementia was divided into four categories: no dementia, AD, vascular dementia, or other dementia.

The data obtained during autopsy included heart weight measurements (I), and the diagnosis of myocardial infarctions (MIs; I) using the naked eye and subsequent confirmation by histological examination. Both acute infarctions and old scars were noted. The histopathological diagnosis of AD (II, III) was set using the National Institute and Reagan Institute (NIA-RI) criteria (The National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the neuropathologic assessment of Alzheimer's Disease) (Hyman and Trojanowski, 1997; Geddes et al., 1997), and the modified NIA-RI criteria as described previously (Polvikoski et al., 2001).

2.1.2. Autopsy with neuropathological examination (II, III)

By December 2000, 306 autopsies (306/601; 51%) with neuropathological examination were performed. In the SSA study (I), the final number of acceptable cases was 256, representing 46% (256/553) of the Vantaa 85+ study population. The CAA study (II) included 74 autopsied individuals, representing 97% (74/76) of all individuals ≥95 years of age. The SSA/CAA study (III) was comprised of all 63 autopsied individuals aged 95 years or more, from which both myocardial and histological autopsy samples and a blood sample for APOE genotyping were available.

At the general autopsy, the heart weight was measured (I), the prescence of amyloid was studied (I), and the diagnosis of myocardial infarctions (MIs) was made with the naked eye (I). Both acute MIs and old scars were noted, and their prescence was confirmed by histological examination. One standard specimen of the interventricular septum of the heart was taken, and two to four additional samples if the macroscopic finding was not normal. One single sample was taken in 65% (167/256), two samples in 26% (66/256), three samples in 8% (20/256), four samples in 0.8% (2/256) and five samples in 0.4% (1/256) of the study subjects, with a mean of 1.5 samples for one individual. The specimens were fixed in 4% phosphate-buffered formaldehyde solution. The histological diagnosis of MIs was based on hematoxylin-eosin stained tissue sections. SSA was diagnosed by detecting amyloid on the Congo red stained, five μm-thick sections, by identifying the typical red–apple green birefringence of amyloid in polarized light. Based on Congo red, the amount of amyloid in the myocardium was semi-quantitatively graded (**Table 8a**): 0 = no amyloid; 1 = small amounts

of amyloid in the vascular walls or between the myocardial cells; 2 = clearly detectable areas of amyloid in several visual fields, including vascular deposits; 3 = large amounts of amyloid.

Table 8a. Grading the severity of SSA.

Congo red staining in the myocardium	Grade of SSA	= Severity of SSA
Negative	0	No
Positive reaction in occasional blood vessels or in small areas between the cardiac myocytes	1	Mild
Clearly detectable reaction in several regions	2	Moderate
Strong reaction in large areas	3	Severe

Note: The diagnosis is based on red-green birefringence in polarized light in a light microscope.

For the CAA study (II), the brains were fixed in 4% phosphate-buffered formaldehyde. Five tissue samples from the frontal, temporal, and parietal lobes, hippocampus, and cerebellum were embedded in paraffin and stained with histological Congo red, hematoxylin and eosin, and iron stains. Grading of the severity of CAA was performed following the guidelines presented by Ellis et al. (Ellis et al., 1996). Eight μm-thick paraffin sections from the frontal, parietal, temporal, cingular, and cerebellar cortex were stained with Congo red and analyzed in polarized light. The Congo red-positive small leptomeningeal and cortical blood vessels were identified and their percentage of all respective vessels was estimated (**Table 8b**). The borders in the samples were set as follows: 0% for no CAA, 1-29% for weak (mild CAA, grade 1), 30-59% for moderate (moderate CAA, grade 2), and 60-100 % for strong positivity (severe CAA, grade 3). In severe CAA, amyloid extended into the surrounding brain parenchyma. A general score of CAA severity (ranging from 0-3) for each individual patient was given, by taking the average of four examined cerebral brain regions (**Table 5**). The cerebellar samples were not included to this score, as these severity scores were later compared with the cognitive status of the subjects. The brains were also studied for cortical or subcortical infarctions, older ischemic scars, or hemorrhages for all cerebral areas mentioned above. The ischemic lesions were diagnosed using hematoxylin and eosin stained five μm-thick sections. Further, to confirm the diagnosis of past hemorrhage, the presence of iron stored in macrophages was evaluated on five μm-thick sections using routine histological iron stain. In this staining, the amounts of blue-stained macrophages and erythrophages with brown cytoplasm were counted, and based on this the grade of hemorrhage (0-3) was assessed. Small lacunar lesions without any tissue reaction were not considered, as it is very difficult to differentiate them from artifactual post-mortal alterations.

Table 8b. Grading the severity of CAA*.

% Congo red-positive blood vessels of small lepto-meningeal and cortical blood vessels in a brain region	Grade of CAA	= Severity of CAA
0	0	No
1-29	1	Mild
30-59	2	Moderate
60-100	3	Severe

Note: The diagnosis is based on red-green birefringence in polarized light in a light microscope.

*The methods follow the guidelines presented by Ellis et al.1996. In addition to the cortical samples (frontal, temporal, and parietal lobes, cingulate gyrus), cerebellum was also studied. An individual CAA severity score was obtained by taking the average of the four cortical regions.

In study II, the neuropathological diagnosis of AD (II) was set using the modified NIA-RI criteria (Polvikoski et al., 2001). The original NIA-RI criteria for neuropathological AD consist of a combination of the CERAD protocol (Mirra et al., 1991) and the Braak staging system (Braak and Braak, 1991). The CERAD protocol is based on estimation of the frequency of neuritic plaques in the cerebral cortex, while Braak staging rests on a hypothesis of progression of neurofibrillar pathology in the brain according to different stages of the disease. The modified classification system (Polvikoski et al., 2001) creates low and high probability groups for AD. Individuals with moderate or frequent neuritic plaques in the CERAD classification, and with stages III to VI in the Braak staging system, were classified as high probability cases for neuropathological AD and categorized as "NIA-RI AD" cases. However, both original and modified criteria can only yield a possibility and not a definite diagnosis for AD.

2.2. AGel amyloidosis (IV)

2.2.1. Clinical examination, laboratory tests, and radiological and neurophysiological studies (IV)

In this study (IV), amyloidosis was diagnosed when the patient was in his fifties. Laboratory studies performed during admission to the hospital included blood cell counts, erythrocyte sedimentation rate, glucose, protein electrophoresis, electrolytes, creatinine, α2-globulin fraction, creatine kinase, aldolase, aspartate and alanine transaminases, γ-glutamyltransferase, folic acid, vitamin B12, syphilis serology, and ECG. Radiological magnetic resonance imaging (MRI) studies on brain, spinal cord, and lower leg skeletal muscles were performed with a 1.5 T high-field unit (Siemens Vision, Erlangen, Germany). Neurophysiological tests included motor nerve conduction,

sensory evoked potential (SEP), needle electromyography (EMG), median nerve stimulation, and quantitative sensory testing (QST).

2.2.2.Autopsy with neuropathological examination (IV)

The brain and spinal cord, peripheral nerve and skeletal muscle specimens were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin, and for sural nerve specimens, also in Epon. Congo red was used to detect amyloid, and immunohistochemistry (IHC) was carried out on five μm-thick tissue sections and processed using the avidin-biotin complex method, to characterize amyloid proteins and associated components.

2.3. Staining methods

2.3.1. Histological staining methods

2.3.1.1. Congo red

Congo red staining was performed using a modified Puchtler's staining method and Congo red purchased from Merck KGaA, Darmstadt, Germany (Kongorot C.1.22120) on five μm- (heart) or eight μm- (brain) thick paraffin-embedded tissue sections. 50 ml of saturated Congo red solution was made by adding the dye to 50 ml of saturated NaCl (in 80% alcohol) solution and 0.5 ml of 1% NaOH. The saturated dye solution was stirred and filtered. Next, the paraffin-embedded tissue sections were deparaffinized in the typical manner, rehydrated, and placed in Mayer's hematoxylin for 10 minutes. After washing twice in distilled water, the sections were incubated for 15 min in the saturated NaCl solution followed by saturated Congo red solution for 20 minutes. Thereafter, the slides were rinsed quickly in 96% ethanol, absolute alcohol, and xylene solutions. In addition to staining tissue slides of the study material, positive and negative control slides were always stained simultaneously.

2.3.1.2. Other stainings

The routine hematoxylin and eosin staining, done using an automated staining device (Labvision), was performed in order to make a morphological diagnosis of the nervous and muscle tissue. A routine connective tissue staining (Herovichi) applied by the staining device was used to diagnose the fibrous scar tissue in the myocardium. A routine Fe++ staining was performed to diagnose recent or prior hemorrhages in the brain tissue.

2.3.2. Immunohistochemistry (IHC)

Immunohistochemical staining methods were used to characterize amyloid and/or other proteins in brain and myocardial tissue. Immunohistochemical

stainings were performed on five µm-thick paraffin sections on all samples, which demonstrated positive reactivity under polarized light after staining with Congo red. The serial sections were processed by the avidin-biotin complex method using Vectastain Avidin-Biotin kit according to the manufacturer's instructions. For control of specificity, normal rabbit serum (Aβ, C3d, TTR), phosphate buffered saline (Aβ), or goat IgG was used (C9). The antigens used are listed in **Table 9**.

2.3.3. Double-staining with medin and Congo red

A double-staining using medin and Congo red was performed on selected cases with severe CAA. Briefly, IHC using rabbit anti-medin antibody (to lactadherin at positions 286-293; A172) was performed using normal goat serum for blocking (microwave, pH 6, primary antibody overnight in 4°C, visualization with DAB). After IHC, Congo red staining was carried out on the same slides. In the light microscope, the Congo red-positive areas showed red-green birefringence in polarized light whereas the structures that were reactive in the anti-medin IHC were detectable as brown granules.

2.4. Genetic analyses (I, II, IV)

2.4.1. Candidate gene polymorphisms (I, II)

Genetic polymorphisms in the following genes were analyzed by PCR-based methods as described earlier: a*ngiotensin converting enzyme* (*ACE)*, *alpha-2 macroglobulin* (*α2M)*, *apolipoprotein E (APOE)*, *beta-amyloid cleaving enzyme2 (BACE2), lipoprotein lipase* (*LPL)*, *low-density lipoprotein receptor-related protein* (*LRP)*, *prion protein*, and *tau* (Myllykangas et al., 1999; Baker et al., 2000; Myllykangas et al., 2000; Nurmi et al., 2003; Myllykangas et al., 2005; Myers et al., 2005).

2.4.2. TTR gene sequencing (I)

The gene for TTR was sequenced in six individuals with severe SSA (grade 3) by amplifying the exons of the TTR gene by standard PCR methods. PCR products were purified using enzymatic ExoSAP treatment (USB Corporation, Cleveland, OH, USA) and sequenced using BigAnalyzed (Applied Biosystems). The sequence analysis was carried out using the Sequencer 4.5 Software (Gene Codes Corporation, Ann Arbor, MI, USA).

2.4.3. Genotyping of the exon 2 mutation in the TTR gene (I)

Genotyping of the exon 2 mutation in the TTR gene was performed in all 256 samples by using the same PCR primers and conditions that were used for the sequencing of exon 2. The PCR product was digested with the restriction enzyme Msp I (New England Biolabs, Ipswich, MA, USA).

Table 9. Antigens used for immunohistochemistry.

Immunogen	Type/Clone	Source/Manufacturer's code nr.	Pretreatment	Dilution	Incubation time	Detection/ Chromogen
AGel subunit	Rabbit anti-IgG	Haltia et al., Am J Pathol 1990	No	1:5000	Overnight*	AEC
α2M	Rabbit polyclonal to human α2M	Abcam, Cambridge, UK/ ab2405	No	1:500	Overnight*	AEC
Amyloid P component	Rabbit anti-IgG	Dako A/S, Glostrup, Denmark	0.1% protease +37°, 2 min	1:500	Overnight*	AEC
Aβ peptide residues 17-24	4G8	Senetek PLC, Maryland Heights, MO	100% formic acid, 5 min	1:1000	Overnight*	AEC
Complement 3d	Rabbit anti-human C3d	Dako, Glostrup, Denmark	**	1:250	**	DAB
Complement 9	Goat anti-human C9	Quidel Corporation, CA, USA	**	1:1000*	**	DAB
LA positions 286-293	A172	Dr. Per Westermark, Uppsala, Sweden	MW, citric acid, pH 6	1:2000	Overnight*	DAB
LCA***	2B11 + PD7/26	Dako, Glostrup, Denmark/ M0701	MW, Tris-EDTA, pH 9	1:1000	30 min	DAB
Phf-tau	AT8; mouse anti-human	Innogenetics, Zwijndrecht, Belgium/ BR-03	pepsin	1:2000	Overnight*	AEC
Skeletal Myosin Heavy Chain (fast)***		Bio-Makor	trypsin	1:4000	30 min	DAB
TTR	Rabbit anti-human TTR	Dako, Glostrup, Denmark	MW, citric acid, pH 6	1:1000	Overnight*	AEC
Tau	Rabbit anti-human tau	Dako, Glostrup, Denmark/ A0024	pepsin	1:800	Overnight*	AEC
SMI311***	Mouse anti-IgG1, anti-IgM	Sternberg Monoclonals Inc., Lutherville, Maryland, USA/ SMI311	MW, Tris-EDTA, pH 9	1:10000	30 min	DAB

AGel = gelsolin amyloid protein; AEC = 3-amino-9-ethylcarbazole; α2M = alpha2-macroglobulin; LA = lactadherin (BA46; milk fat globule protein); MW = microwave; LCA = common leukocyte antigen (CD45); DAB = diaminobenzidine; TTR = transthyretin; SMI311 = pan-neurophilament protein antibody cocktail. *At room temperature. **Available upon request. ***Used automated staining device (Labvision).

2.4.4. *APOE* genotyping (I, II)

APOE genotyping was performed as described previously (Myllykangas et al., 2002). Because very advanced age may influence the relative frequency of different *APOE* genotypes and the risk for amyloidotic cerebral diseases, the frequencies of *APOE* alleles and genotypes in the very elderly subgroup (I, II) were first compared to another age group of the study population, i.e. those who died at the age of 85-94 years old (n=400). The frequency of the ε4 allele was slightly reduced in the oldest age group (12.7% vs. 17.1%), but this difference was not statistically significant. None of the alleles or genotypes of *APOE* differed significantly between these subgroups of the Vantaa 85+ study population.

2.4.5. Gelsolin gene analysis (IV)

Genetic testing for the gelsolin gene mutation was performed as described earlier (Paunio et al., 1992).

2.5. Statistical analyses (I, II, III)

The statistical analyses were performed using the SPSS for Windows version 12.0.1 software. Differences between the groups (SSA+/SSA-, CAA+/CAA-, genetic polymorphisms, diabetes, hypertension, BMI category, smoking; dementia, dementia type; I, II, III) and interrelationships between the variables (CAA, APOE, and dementia; II) were analyzed with the Chi-Squared or exact test for linear categorical variables. For continuous variables (age at death, heart weight; I), logistic regression analysis (I) or Student's two-tailed t-test (age at death; III) was used. The association between genotype and SSA was studied by logistic regression, controlling for possible confounding effect of age and MI on (I). Correlation between the genotypes and severity of SSA (I) was conducted using the Chi-Squared or exact test for linear trend. P-values <0.05 were considered significant.

3. APPROVAL FOR THE STUDY

The Vantaa 85+ study was approved by the Ethics Committee of the Health Centre of the City of Vantaa. The Finnish Health and Social Ministry has approved the use of the health and social work records, and death certificates. Blood samples were collected only after the subjects or their relatives gave informed consent. The National Authority for Medicolegal Affairs has approved the collection of the tissue samples at autopsy as well as their use for research. A written consent for autopsy was obtained from the nearest relatives.

RESULTS:

1. FREQUENCY AND DISTRIBUTION OF SSA AND CAA (I, II, III)

1.1. Frequency of SSA (I)

1.1.1. Frequency of SSA in a population aged 85 years or more (I)

Amyloid deposition in one or more of the Congo red stained myocardial specimens (**Figure 1A,B;** p.28) was detected in 25% (63/256) of the study (I) population. In all cases, the amyloid deposits showed a positive reaction in anti-TTR immunohistochemical stainings (**Figure 1C**). SSA was mild in 78% (49/63), moderate in 11% (7/63), and severe in 11% (7/63).

1.1.2.Frequency of SSA in a population aged 95 years or more (III)

SSA was found in 37% (23/63) of individuals aged 95 years or more (III), and consisted of 19 women and four men.

1.1.3. Distribution and morphology of SSA in the myocardium (Tanskanen et al., unpublished data)

Congo red positivity was observed in every specimen taken from the myocardium in almost all of the subjects (97%; 61/63) having SSA. Among individuals positive for Congo red in the myocardium, there was only one in whom amyloid was solely vascular. Out of the 62 individuals with parenchymal SSA, in 52% (32/62) vascular amyloid could be detected as well. Histologically, the amyloid distribution in the myocardium was patchy and haphazard. In the tissue sections, the TTR-based amyloid deposits were located between the preserved striated muscle fibers. Sometimes, but not always, they were located in proximity to fibrous scars (**Figure 1**), acute infarctions, or blood vessels. In contrast, in the fibrous areas, the deposits were relatively small. Amyloid deposits were not associated with any particular anatomical region of the heart. In many cases of moderate or severe SSA, the myocardial cells showed increasing size and deformity of the nuclei, typical of chronic hypoxia. Occasionally, in a few of the control (Congo red negative) cases, minute reactivity in the anti-TTR IHC was observed.

In the histological connective tissue (Herovichi), connective tissue staining (**Figure 1D**; p.28) was performed on 14 cases with moderate or severe SSA, and amyloid deposits could be readily distinguished as light orange homogenous material, clearly distinct from the red-stained fibrous scar tissue and/or other structures. In contrast, in routine hematoxylin and eosin staining (**Figure 1E**; p.28), the amyloid material could be missed.

1.1.4. Distribution and morphology of TTR-based amyloid in the pulmonary tissue (Tanskanen et al., unpublished data)

In this series of 256 individuals, pulmonary tissue samples were examined in 164. Among these, only two subjects (1%) had detectable amyloid in blood vessels in the lung tissue but not in the heart.

1.2. Frequency of CAA (II, III)

1.2.1. Frequency of CAA in a population aged 95 years or more (II, III)

In study II, Congo red positivity (**Figure 2A,B;** p.34) was detected in histological analyses of frontal, parietal, temporal, cingular, and cerebellar cortical specimens from 36 (36/74; 49%) of the 74 study subjects, in altogether 99 (99/370; 27%) of the tissue samples analyzed. On average, three brain regions were involved. The Congo red-positive blood vessels were also reactive in the anti-Aβ IHC (II: **Figure 3b**; III: **Figure 1**). In a smaller subset of individuals (III, n=63), CAA was detected in 28 (44%) of the 63 individuals, where the prevalence was 42% (22/53) in females and 60% (6/10) in males.

1.2.2. Distribution of CAA in different brain regions (II)

CAA was most prevalent in the frontal cortex (30/99) and least frequently observed in the cerebellum (8/99). The score assignment for CAA severity was 1 in 22 (61.1%), 2 in 11 (30.6%), and 3 in 3 individuals (8.3%). Severe CAA was observed in 24 of 99 specimens (24%), and was most often located frontally (9/24, 37.5%). Mild CAA was the most common form, observed in 64% of the specimens (64/99). A frequency of occurrence was comparable in the frontal (16/64), temporal (16/64), and parietal lobes (15/64). Moderate and severe CAA was found in 11% and 25% of the specimens, respectively (II: **Figure 1**). The amyloidotic blood vessels were generally devoid of leukocytes. The presence of leukocytes in CAA is rare. In addition to the cases where small infarction were associated with CAA (**Figure 2A-D**; p.34), scattered CD45-positive cells (leukocytes) were detected in a few samples with severe CAA.

2. ASSOCIATION BETWEEN SSA AND CAA (III)

The study investigating the association between SSA and CAA was performed on 63 of the subjects aged 95 years or older, including 53 females and 10 males, for whom both heart and cerebral specimens were available. Nine individuals (14%; seven women, two men) had both SSA and CAA, in contrast to 21 (33%; 19 women, two men) who neither had SSA nor CAA. Thus, 67 % of the subjects (42/63) had SSA, CAA, or both. In the statistical analyses, SSA was not associated with CAA (p=0.43 for all, 0.41 for women). Of the nine individuals with both SSA and CAA, both were grade 1 in four individuals. In two subjects, SSA grade 1 co-occurred with CAA grade 2-3, and in another two subjects, CAA grade 1 co-occurred with SSA grade 2-3. In one individual, both SSA and CAA were scored as grade 2-3.

3. CLINICAL FEATURES AND AUTOPSY FINDINGS IN SSA, CAA, AND AGEL AMYLOIDOSIS (I, II, III)

3.1. Age (I, II)

3.1.1. Age and SSA (I)

Characteristics of the SSA study (I) population and the number of individuals with or without SSA are shown in **Table 10**. The proportion of individuals with SSA clearly increased with increasing age at the time of death of the subjects. SSA was detected in 17% (11/65) of the individuals who died at the age of 85-89.9 years old, 23% (29/127) of those who died at the age of 90-94.9 years, 32% (18/56) of those who died at the ages of 95-99.9 years, and 63% (5/8) of those reaching at least 100 years of age. The results reveal that SSA is significantly associated with the age at death (OR 1.13; 95% CI 1.05–1.22; p=0.002).

3.1.2. Age and CAA (II)

Characteristics of the CAA study (II) population is illustrated in **Table 10.** In statistical analyses using linear regression, age was not associated with CAA (p=0.974).

Table 10. Demographic and health-related characteristics of individuals with (+) or without (-) senile systemic amyloidosis and cerebral amyloid angiopathy.

	Study I	SSA+	SSA-	Study II	CAA+	CAA-
N	256	63	193	74	36	38
Age, yr*	92.5 ± 3.8	93.8 ± 4.3	92.0 ± 3.5	96.6		
		p=0.002[a]				
Men, %	16	22	15	18	22	11
BMI*,**	3.0± 0.7	2.85 ± 0.7	3.05 ± 0.7			
DM, %	22	21	23		3	20
						p=0.021[b]
Hypertension, %	29	31	28			
Smoking***, %	16	21	14			
Dementia, %	65	52	73	66	81	53
						0.01
Heart weight, g*	356.6 ± 89.5	381.7 ± 106.6	348.8 ± 81.9		320.8±63.4	377±101.6
		p=0.015[a]				p=0.017[b]
MI, %	40	50	37		29	44

SSA = senile systemic amyloidosis; CAA = cerebral amyloid angiopathy; SD = standard deviation; BMI = body mass index = weight divided by height squared.

*Mean ± SD.

**BMI values were classified into five categories: 1 ≥ 32; 2 = 28-31; 3 = 19-27; 4 = 15-18; 5 ≤ 14

***In 1991.

[a]When comparing the subjects with (+) or without (-) SSA.

[b]When comparing the subjects with (+) or without (-) CAA.

3.2. Gender (I, II)

3.2.1. Gender and SSA (I)

The proportion of males in the SSA study (I) was 17% (43/256) and 83% (213/256) for females. Of all subjects having SSA, 22% (14/63) were males compared to 15% (29/193) in the SSA population (**Table 10**). The overall frequency of SSA did not associate with gender. Severe (grade 3) SSA occurred in 10% (4/43) of the males compared to 1% (2/213) in females, indicating significant association between severity of SSA and the male gender (p=0.022).

3.2.2. Gender and CAA (II)

The population in the CAA study (II) included 13 males and 61 females. Out of the 36 individuals having CAA, 22% (8/36) were males and 78% (28/36) females whereas 11% (4/38) were males in the controls (CAA subjects). Among all 36 subjects with CAA, it was severe in 25% (2/8) of the males

compared to only 3% (1/28) of the females. Statistically, gender was not associated with CAA (p=0.237).

3.3. Health-associated risk factors (I)

3.3.1. BMI (body mass index), diabetes mellitus (DM), hypertension (HT), smoking, and SSA (I)

The mean BMI (body mass index = weight divided by height squared; classified into five categories: 1 = ≥ 32; 2 = 28-31; 3 = 19-27; 4 = 15-18; 5 ≤ 14) values for individuals with SSA (n=256, study I) were 2.85 ± 0.7, compared to 3.05 ± 0.7 for those without SSA (**Table 10**). Low BMI showed a non-significant tendency towards association with SSA (p=0.053). SSA was not associated with DM, HT, or smoking (**Table 10**).

3.3.2. BMI, DM, HT, smoking, and CAA (Tanskanen et al., unpublished data)

DM was detected in one individual (1/35; 3%) of those having CAA, in contrast to eight individuals (8/40; 20%) from those not having CAA (**Table 10**). The proportion of non-diabetics was 97% (34/35) in the CAA-positive population and 80% (32/40) in the CAA-negative population. There was significant association between lack of DM and CAA (p=0.021). CAA was not associated with BMI, HT, or smoking (**Table 10**).

3.4. Clinical dementia (II, III)

3.4.1. Dementia and SSA (III)

The association between dementia and SSA was studied in the 63 subjects (study III, 53 women, 10 men) aged 95 years or more. In this population, 36% of the subjects (23/63; 19 women, four men) had SSA. Clinical dementia was stated in 65% (41/63; 36 women, five men). Of the 23 SSA+ individuals, dementia was clinically diagnosed in 52% (12/23; ten women, two men). Out of the 12 demented SSA+ individuals, 67% (8/12) had clinical AD (six women, two men) and 33% (4/12, four women) had other types of dementia. There were 40 individuals without SSA, in whom clinical dementia was diagnosed in 73% (29/40; 26 women, three men). In the subjects without SSA, AD was diagnosed in 55% (16/29;15 women and one man) and other dementias in 45% (13/29; 11 women and two men). In the statistical analyses, SSA was not associated with clinical dementia, AD, or other dementias. In both groups of individuals (SSA+ and SSA-), the same number of individuals (11) had normal cognition.

3.4.2. Dementia and CAA (II, III)

In study II, 66% (49/74) of the 74 study participants were demented. Out of the 36 individuals having CAA in this study, dementia was stated in 81% (29/36). Out of the 29 demented patients with CAA, AD was clinically diagnosed in 62% (18/29), vascular dementia in 24% (7/29), and other dementias in 14% (4/29). Of the 49 clinically demented individuals, 59% (29/49) had CAA with 26% (13/49) having moderate or severe CAA (grades 2 or 3). CAA occurred in 28% (7/25) of the 25 non-demented individuals, but only one subject had grade 2 or 3. CAA was significantly associated with clinical dementia (p=0.01; II: **Table 1c**), particularly moderate or severe CAA (p=0.003; study II). However, dementia was not associated with the severity of CAA (p=0.22; II: **Table 1d**).

Study III examined a somewhat smaller subset of the most elderly population. In this study, the results for dementia and CAA were in line with study II, as dementia was stated in 79% (22/28) of the individuals having CAA and only 59% (19/37) of those not having CAA.

3.5. Clinical, laboratory, and radiological findings in AGel amyloidosis (IV)

At age 78, the patient had a facial appearance characteristic of AGel amyloidosis, dysarthria, facial myokymic diplegia, corneal areflexia, and atrophy of muscles of the face, neck, and shoulders. Minor muscle weakness and atrophy in the proximal lower and distal upper limbs, and sensory impairment in the limbs and trunk were stated. The tongue was atrophic with an enlarged base. Deep tendon reflexes were abolished, and plantar responses were flexor. The main clinical problem was a severe ataxia such that standing and sitting upright were difficult. All laboratory tests were normal apart from minor blood leukocytosis (10.4 E9/L) and a slight elevation of the α2-globulin fraction (9.3 g/L; normal 4.5-8.9 g/L). The G654A gelsolin gene mutation was diagnosed by solid-phase minisequencing (Paunio et al., 1992a). Radiologically, moderate brain and spinal cord atrophy with occasional unspecific foci in the cerebral white matter occurred. The images also reveal atrophy and fatty degeneration of the multifidus and longissimus dorsi muscles of the back and less pronounced atrophy of the hamstring muscles of the thigh. In the neurophysiological studies, the motor nerve conduction velocities were slightly abnormal with low amplitude responses. The sensory response in the sural nerve was absent and its amplitude in the radial nerve low. The F-response of the posterior tibial nerve was delayed. In the needle EMG, severe generalized chronic axonal neuropathy with loss of motor unit potentials (MUPs) occurred. Long duration, high amplitude polyphasic MUPs were seen in all limb and trunk muscles. In the facial muscles, total paralysis was found with only single MUPs detected in the

orbicularis oculi muscle. Severe MUP loss also occurred in the lingual, masseter and platysma muscles. At somatosensory evoked potential (SEP) examination, no peripheral- or thalamocortical responses were able to be elicited at median nerve stimulation. In quantitative sensory testing (QST), the sensations of vibration, as well as cold and warm temperature in upper and lower extremities were abnormal. Pain sensation for cold was normal, but heat pain sensation was abnormal in the hypothenar area.

3.6. General autopsy findings (I)

3.6.1. Heart weight and myocardial infarctions (MIs) in SSA (I)

In 50 % of the histological samples from individuals with SSA, acute MIs or older scars were detected compared to 37% in those without SSA (**Table 10**). In the Chi-Squared or exact test for linear categorical variables, the MIs showed a nonsignificant tendency towards association with SSA (p=0.076). However, in the logistic regression analysis (I: **Table IV**), after controlling for confounding factors, a significant association between SSA and MIs was detected (p=0.004).

The mean weight of the heart was 382 ± 107g in subjects with SSA, and 349 ± 82g in those without SSA (n= 256; **Table 10**). The difference between the groups was statistically significant (OR=1.004; 95% CI 1.001–1.007; p=0.015). In addition, the weight of the heart was very strongly associated with MI (p=0.000).

3.6.2. Heart weight and MIs in CAA (Tanskanen et al., unpublished data)

The mean weight of the heart in individuals with CAA was 320.8g (SD= 63.37, SE= 11.57), and 377.4g (SD= 101,57, SE= 17,18) in those without CAA (**Table 10**). Increased heart weight associated significantly with the absence of CAA (p=0.017). Nine (29%) old scars or acute infarctions were detected in the cardiac samples of individuals having CAA, compared with 16 (44%) in those without CAA. Statistically, the difference between these groups was not significant.

3.7. Neuropathological findings (II, III, IV)

3.7.1. Cerebral infarctions and hemorrhages in CAA (II)

The presence of cortical ischemic lesions or hemorrhages was studied in 74 individuals aged 95 years or older (II). Twelve small cortical infarcts, scars, or small anoxic lesions were found in 11 samples (12/99; 12.1%) from

nine individuals with CAA (9/36; 25%). Anatomically, these small vascular lesions were located closely to the amyloidotic blood vessels (**Figure 2A-D**; p.34). Seven of these CAA+ individuals had clinical AD, one had vascular dementia, and one was not demented. A large acute infarction was stated in one individual without CAA (1/38; 3%).

In 13 individuals with CAA (13/36; 36%), iron-positive macrophages and erythrophages indicating earlier minor blood extravasation or hemorrhage (**Figure 2F**; p.34) occurred in 22 samples (22/99; 22%). In all samples, the amount of iron was minor. No major hemorrhages were found. In 17 individuals without CAA (17/38; 45%), 22 iron-positive samples (22/190; 12%) were detected.

3.7.2. Neuropathological AD and CAA (II, III)

Senile plaques and neurofibrillary tangles represent the neuropathological hallmarks of AD. In the present study (II), the presence of CAA was compared to the presence of histopathological AD findings according to the NIA-RI criteria of AD pathology described earlier (Polvikoski et al., 2001). Out of the 36 individuals having CAA, clinical dementia was stated in 29 (29/36; 81%). In histological samples from these 29 demented individuals, 22 subjects (22/29; 76%) fulfilled the histopathological criteria of NIA-RI AD**.** Out of the 38 subjects not having CAA, 20 were demented (20/38; 53%) and nine (9/20; 45%) had neuropathological AD. Statistically, CAA was significantly associated with NIA-RI AD (p=0.0005; II: **Table 2a**). Interestingly, a subset analysis of CAA+ subjects also showed that CAA was associated with clinical dementia in subjects without NIA-RI AD pathology (II: **Table 2b**), and thus the association between CAA and clinical dementia did not depend upon histopathological AD in this age group.

3.7.3. Neuropathological AD and SSA (III)

In study III, only four individuals out of the 23 subjects with SSA had frequent plaques using the CERAD criteria for neuropathological AD (**Figure 3E**), and four had neurofibrillar stage 5-6 in the Braak staging system for neuropathological AD (**Figure 3F**). The corresponding numbers in the 40 subjects without SSA were three and nine, respectively (**Figure 3 E,F**). In the statistical analyses, SSA was not associated with neuropathological AD.

3.7.4. Neuropathological findings in advanced AGel amyloidosis (IV)

Neuropathological examination revealed marked gelsolin amyloid deposition at vascular and connective tissue sites along the entire length of the peripheral nerves extending to the spinal nerve roots, associated with severe degeneration of affected muscle tissue (IV: **Figure 1d**), nerve fibers and the posterior columns (IV: **Figure 1e**). In the case of advanced AGel amyloidosis, the loss of myelinated axons and reduced density of neuronal

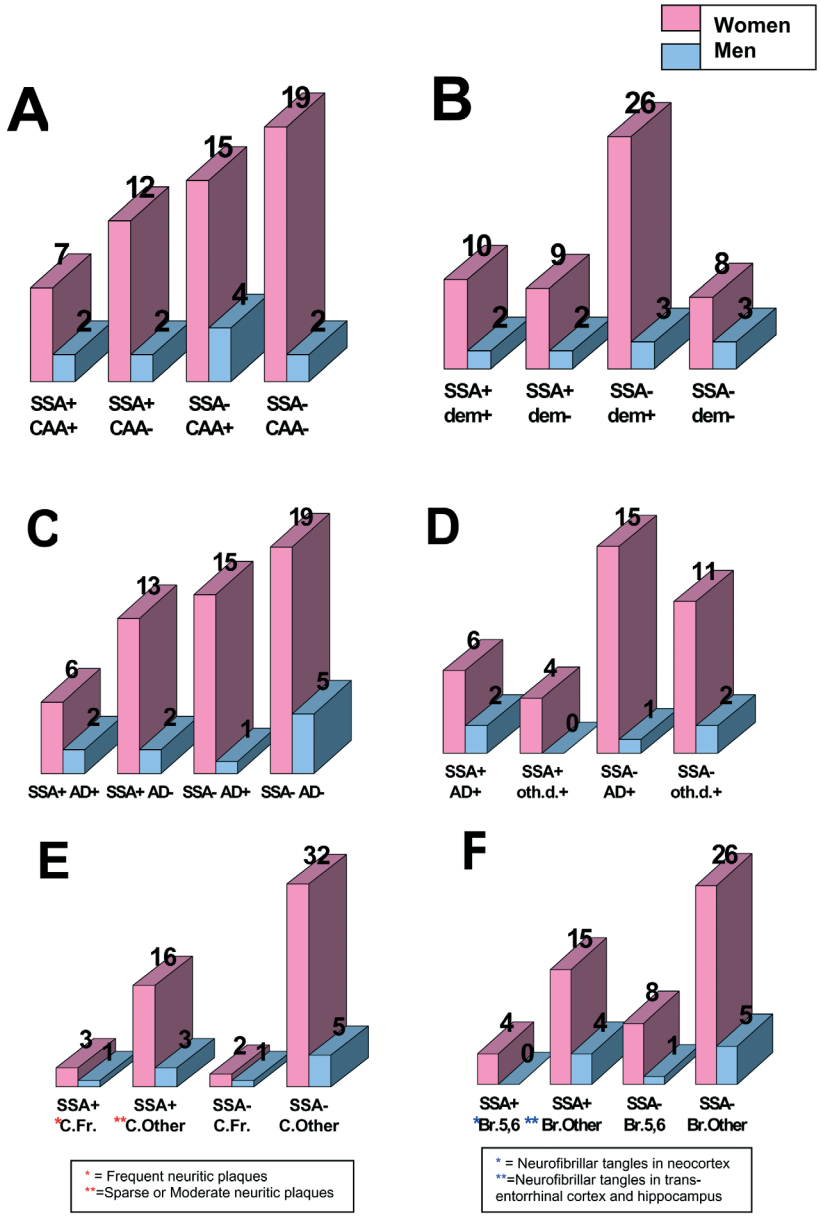

Figure 3.

Number of individuals with or without SSA, and with or without CAA (**A**), clinical dementia (**B**), clinical AD (**C**), other dementia (**D**), frequent neuritic plaques according to CERAD criteria (**E**) and neocortical neurofibrillar tangles (**F**).

Abbreviations: **dem** = dementia; non-AD = other types of dementia than AD; **C.F** = frequent neuritic plaques corresponding to class C for individuals aged >75 years in CERAD classification; **C.s,m** = no, sparse or moderate plaques, corresponding to class 0, A, or B for individuals aged >75 years in CERAD classification; **Br.5,6** = Braak stages 5 and 6, corresponding neocortical neurofibrillar stage; **Br.0.4** = Braak stages 0-4, corresponding to no, entorhinal or limbic neurofibrillar stages.

cell bodies in the hypoglossal nerve nucleus was clearly detectable by using SMI311, a pan-neurophilament protein antibody cocktail. Myosin Heavy Chain fast antibody, which stains the type 2 fibers of skeletal muscle while leaving the type 1 fibers unstained, was used to specify the type of muscle atrophy in the patient with advanced AGel amyloidosis. In this patient, both the stained type 2 fibers as well as the unstained type 1 fibers were atrophic, indicating a combination of inactivity and denervation atrophy (IV: **Figure 1d**). In the brain, some cerebral blood vessels showed amyloid that reacted in anti-variant gelsolin IHC. Numerous diffuse, but only a few neuritic plaques and neurofibrillary tangles, were seen in the cerebral cortex.

4. GENETIC POLYMORHISMS IN SSA AND CAA (I, II)

4.1. Genetic polymorphisms in SSA (I)

The results of the genetic association studies in SSA and CAA are shown in **Table 11**. SSA associated significantly with the single nucleotide polymorphism (SNP) G/G genotype in exon 24 of the *α2M* gene (p=0.042). This polymorphism also showed significant association with the severity of SSA (p=0.012). Further, the exon 9 deletion/insertion (H1/H2) polymorphism of the *tau* gene was significantly associated to SSA (p=0.016), as well as the severity of SSA (p=0.003). In order to find out if the *TTR* gene itself was associated with SSA, the coding regions of the *TTR* gene were sequenced in six individuals with grade 3 SSA. This analysis showed the predicted wildtype sequence in five cases, but one individual had the A/G genotype in exon 2 of the *TTR* gene. This SNP, denoted rs1800458, can be found in the NCBI database (http://ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp) and is predicted to result in a non-synonymous amino acid change (Gly26Ser). This SNP was subsequently analyzed in all 256 study. Two homozygous (Ser/Ser) and 47 heterozygous (Gly/Ser) individuals were found, while 188 subjects were homozygous for the wildtype allele (PCR failed in 19 samples). The allele frequency was 0.11, and there was no significant difference between the allelic frequencies in individuals with, or without, SSA.

SSA was not associated with the exon 18 polymorphism of the *α2M* gene, the *tau* RS3758, RS24717, and RS7521 polymorphisms, *APOE* genotype, nor the *ACE*, *LPL*, *LRP*, or *Prion protein* polymorphisms.

Table 11. The results of association analyses comparing the genotype distributions of subjects with or without senile systemic amyloidosis (SSA) and cerebral amyloid angiopathy (CAA).-

Gene/ polymorphism	p-value/SSA[a]	p-value/CAA[b]
Angiotensin converting enzyme		
Insertion-deletion	n.s.	n.s.
Alpha2-macroglobulin		
Exon 24 (Ile1000Val)	0.042[*]	n.s.
Intron17/Exon 18 (5 bp del)	n.s.	n.s.
Apolipoprotein E		
ε2/3/4	n.s.	0.01[**]
Promoter -219	n.s.	n.s.
Promoter -491	n.s.	n.s.
Intron IE1	n.s.	n.s.
Beta-amyloid cleaving enzyme2		
Intron 1a	n.s.	n.s.
Intron 1b	n.s.	n.s.
Exon 6	n.s.	n.s.
Exon 8	n.s.	n.s.
Lipoprotein lipase		
Ser447Ter	n.s.	n.s.
Asn291Ser	n.s.	n.s.
Low-density lipoprotein receptor -related protein		
Exon 6	n.s.	n.s.
Prion protein gene		
C129 M/V	n.s.	n.s.
Tau		
H1/H2	0.016[***]	n.s.
RS3785883	n.s.	n.s.
RS2471738	n.s.	n.s.
RS7521	n.s.	n.s.
Transthyretin		
RS18004	n.s.	n.s.

SSA = senile systemic amyloidoisis; CAA = cerebral amyloid angiopathy; n.s. = not significant.

[a]When comparing subjects with and without SSA.

[b]When comparing subjects with and without CAA.

[*]Chi-squared test for linear trend.

[**]Chi-squared test.

[***]Exact test for linear trend.

4.2. Genetic polymorphisms in CAA (Tanskanen M et al., unpublished data)

One copy of *APOE* ε4 was found in 15 (45%) subjects with CAA compared with three (8%) individuals without CAA ($\chi 2=13.17$, 1 df, $p=0.0003$). No association with ε2 was found. In addition, moderate to severe CAA was associated with *APOE* ε4 ($p=0.03$) but not mild CAA. CAA was not associated with polymorphisms in the following genes: *α2M*, *tau*, *TTR*, *ACE*, *LPL*, *LRPR*, or *Prion protein* (**Table 11**).

5. IHC FOR AMYLOID-ASSOCIATED AND OTHER PROTEINS (I, II, IV)

5.1. Amyloid proteins and Congo red (I, II, IV)

All Congo red-positive structures in the myocardium (I) reacted in the anti-TTR IHC. Similarly, all Congo red-positive cerebral cortical blood vessels (II) reacted in the anti-Aβ IHC, and the amyloid deposits in the patient with AGel amyloidosis (IV) reacted with anti-variant gelsolin IHC. In all types of amyloid, the immunohistochemical reaction was slightly broader than the Congo red positivity.

5.2. AMed amyloid and CAA (Tanskanen et al., unpublished data)

In the medin study, Congo red staining detected CAA in nine out of the ten (90%) individuals with clinical AD. In one subject, there was no CAA in spite of moderate amounts of plaques. Among the nine individuals having CAA, it was mild in five, moderate in two, and severe in two subjects. In all subjects, vascular amyloid deposits reacted in Aβ IHC. The number of senile plaques in Aβ IHC ranged from sparse to frequent. In the seven clinically non-demented (control) individuals, there was no CAA and the Aβ IHC showed sparse senile diffuse, non-amyloid plaques in three subjects. In all cases, varying positivity in the anti-medin IHC was observed in the small and middle-sized arteries in the cerebral cortex and leptomeninges. However, positive reactivity was located outside the amyloid deposits. The finding became clearly evident when double staining with Congo red and medin was performed in cases of severe CAA with total damage of the arterial media. In the amyloidotic vessels, no reaction in the anti-medin ICH was noted.

5.3. Complement (C) proteins in CAA and AGel amyloidosis (II, IV)

5.3.1. C3d and C9 in CAA (II)

In CAA, most amyloidotic blood vessels showed reactivity for antibodies against C3d and C9 (II: **Figure 1c,d**). Reactivity in the anti-C3d IHC was detected in 89% (88/99) of the samples, and 84% (83/99) of the samples in the anti-C9 IHC. In all C3d/C9-negative samples, CAA was very mild (grade 1) with only 1-2% of blood vessels staining positive for Congo red or Aβ. Most complement-negative samples were negative in both complement stainings. The 11 samples with CAA and small cortical infarct(s) showed a less pronounced reaction for C3d and C9 than samples with similar CAA but without infarcts. No difference in IHC reactivity between C3d and C9 was seen when comparing CAA cases with or without iron. The IHC reactivity for C3d and C9 were highly congruent in the examined samples and co-localized with the congophilic, birefringent and Aβ-positive amyloid deposits (II: **Figure 1c,d** and **Figure 2a,b**).

5.3.2. C3d and C9 in AGel amyloidosis (IV)

The histological samples from the patient with advanced AGel amyloidosis showed positive reactions in the anti-C3d and anti-C9 IHCs. The reaction co-localized with Congo red and the anti-variant gelsolin IHC in the neural (IV:**Figure 1h,j**) and vascular structures.

5.4. Alpha2-macroglobulin (α2M) in the myocardium (Tanskanen et al., unpublished data)

Immunohistochemical staining using an antibody against *α2M* was performed on paraffin-embedded myocardial samples from 14 individuals with grade 2-3 SSA. The anti-α2M IHC was negative within myocardial amyloid deposits, but in contrast, the fibrous scar tissue in these samples was stained (**Figure 1F**). In addition, a positive reaction was seen in leukocytes present in the blood vessels.

5.5. Tau and phosphorylated tau in the myocardium (Tanskanen et al., unpublished data)

The anti-tau and anti-phosphorylated tau IHC was negative in the myocardial tissue samples.

6. EFFECTS OF CLINICAL, PATHOLOGICAL, AND GENETIC FACTORS ON SSA AND CAA (I, II, III)

6.1. Multivariate analysis of the effect of MIs, age, and genetic variants of *α2M* and *tau* on SSA (I)

The association between SSA and *α2M* and *tau* was studied using logistic regression analysis to control for the possible confounding effects of age and MIs. The p-values for *α2M* and *tau* remained significant when age at the time of death and MIs were included in the analysis, implicating that the effects of *tau* and *α2M* genotypes were independent of the other variables (I: **Table 4**).

6.2. Association between dementia, CAA, *APOE* genotype, and histopathological AD (II)

CAA associated significantly with *APOE* genotype (p=0.0004), largely due to the increased frequency of ε3/4 and decreased frequency of the ε3/3 genotype in CAA. CAA also associated with dementia (p=0.01). However, dementia was not associated with *APOE* ε4 in this sample of individuals (p=0.23). Interestingly, the association between dementia and CAA was contributed to by both the ε4+ (p=0.01) and ε4- subjects (p=0.06). We tested whether CAA represents AD pathology in this group of individuals. Indeed, there was also a significant association between CAA and histopathologically-defined AD according to the NIA-RI criteria (p=0.0005). However, the subset analysis of CAA+ subjects suggests that CAA also associates with clinical dementia in subjects without NIA-RI-AD pathology. In conclusion, the association between dementia and CAA depends on neither *APOE* nor histopathological AD in this very elderly Finnish population.

6.3. Association between dementia, SSA, and CAA (III)

Dementia was more common in individuals without SSA (73%) than in those with SSA (52%). CAA was common in demented individuals (54%), both in those with (58%) or without SSA (52%). In contrast, CAA was more seldom in individuals with normal cognition (28%), and this was independent of having SSA (18%) or not (36%) (**Figure 4**).

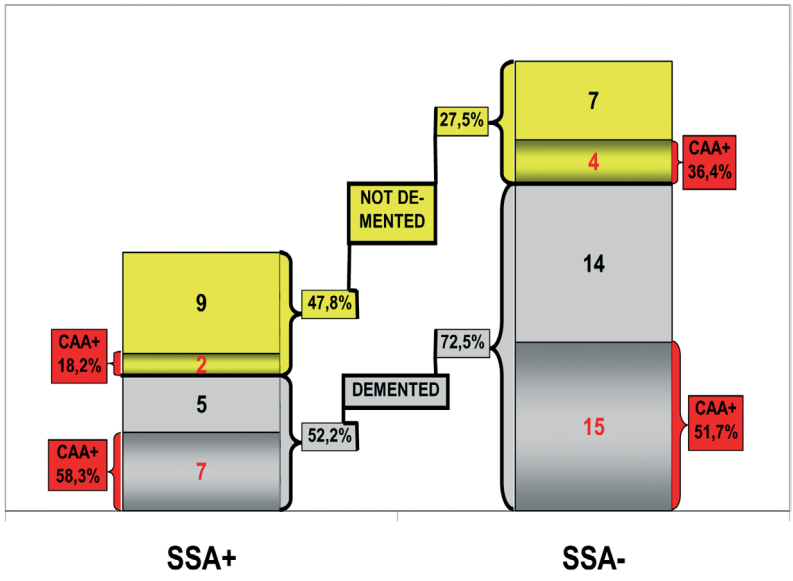

Figure 4.

The number of demented (grey columns) and non-demented (yellow columns) individuals in SSA+ and SSA- groups. In both groups, CAA (red numbers) is common in the demented, but uncommon in individuals who are not demented .

DISCUSSION:

1. FREQUENCY AND DISTRIBUTION OF SSA AND CAA

In previous hospital-based series, SSA has been encountered in 25-28 % of autopsied subjects aged 80 years or over (Cornwell et al., 1983; Westermark et al., 2003). In the present population-based study (I), the prevalence of SSA was 25%, confirming SSA is common in an elderly population. In the present study, the standard myocardial sample was taken from the septum of the heart, a commonly used biopsy site for general autopsy. In other studies, the samples were taken from the "left ventricle" (Johansson and Westermark, 1991) or as "one large transverse section of ventricles" (Sawabe et al., 2003). Here, except for two individuals, SSA was detectable in all myocardial specimens taken. Based on this, one histological sample seems to be sufficient for the diagnosis of SSA. Apart from lack of awareness of SSA, other reasons for SSA being a rare histopathological diagnosis in general autopsy reports may include the presumably benign nature of SSA as well as the common use of hematoxylin-eosin (HE) staining in which the differentiation between amyloid material and fibrous tissue is more difficult. In addition to Congo red, amyloid can be detected and distinguished from fibrous scar tissue by using connective tissue stains. In every case, IHC is necessary in order to type the amyloid. In contrast, HE staining may be more informative in visualizing nuclear morphology, which can be helpful in diagnosing several common conditions potentially leading to death, e.g. malignant neoplasms.

Here, the previous findings showing patchy distribution of SSA in different regions of the heart and the distribution in the individual myocardial samples (Cornwell et al., 1983; Westermark et al., 2003) was confirmed. Thus, the type of distribution of the SSA resembles the patchy distribution of CAA, reported previously (Ellis et al., 1996; Yamada, 2000) and even in the present study (II). SSA is a systemic disorder, and the haphazard distribution of amyloid in the tissue may be due to the the blood borne origin of the amyloid precursor protein, TTR. In addition, the predilection sites of amyloid deposition in SSA (heart and lung) might be due to the abundant vascularity that is typical to these organs. The myocardial amyloid deposits were not associated with scars, infarctions, anatomical regions, or even blood vessels in the tissue sections. However, the present finding is not conclusive, as the conventional stains

may not reliably detect the smallest blood vessels. IHC for endothelial cells (CD131, CD134), preferentially combined with serial sections perpendicular to the blood vessels, would be more informative.

From several patients, there were pulmonary samples available that could be stained with Congo red. Interestingly, only 1 % of the subjects tested positively in the lungs but not in the heart. Thus, determining the occurrence of SSA by solely examining cardiac samples seems to be a reliable method.

CAA (II) was encountered in nearly half of the most elderly population, similar to a previous population-based study (Masuda et al., 1988). In other studies, the frequency of CAA has been shown to increase with increasing age of death of the subjects (Yamada et al., 1988; Masuda et al., 1988; Xu et al., 2003). The occurrence of CAA was not associated with age. However, the finding may not be conclusive as the number of patients in the CAA study was small, the patients represented a very specific age group, and finally because of their narrow age. The study (II) also clearly showed the patchy spatial distribution of CAA, ranging from one to five brain regions. The preferred site was the frontal cortex, as noted earlier (Masuda et al., 1988; Ellis et al., 1996; Yamada, 2000). In other reports, the occipital and parietal lobes have also been prevalent (Vinters and Gilbert, 1983), but unfortunately as in the study by Ellis et al. (Ellis et al., 1996), parietal lobe samples were not available here.

In this work, the methods used by Ellis et al. (Ellis et al 1996; **Table 5**, p.33; **8b**, p.52) were chosen to assess the severity of CAA, for several reasons. By using these methods, several cortical regions can be studied, and clear guidelines are given on how to create an individual CAA score. Both of these aspects are essential in order to correlate CAA with clinical dementia. Secondly, comparing the severity of CAA with the amount of complement proteins necessitates that these two parameters are graded using the same system. Furthermore, the simple four-step semi-quantitative grading system used by Ellis et al. (grades 0 to3) is easy to apply to immunohistochemical complement stainings. The other methods (**Table 5**; p.33) were in some respects inadequate for this work: the method described by Vonsattel (Vonsattel et al., 1991) does not offer individual CAA scoring, and Olichney (Olichney et al., 1995) uses a complex five-step grading system. Thal (Thal et al., 2002), although precisely describing the findings in different blood vessel types, uses a simplified three-step grading system with minor attention to the extent that cerebral regions are affected.

Finally, the present study (III) shows for the first time the high frequency of SSA and CAA together, as either one or both of these conditions were present in almost two-thirds (see Results, Chapter 2) of the very elderly Finnish population. This finding highlights the potential clinical significance of these diseases, as at least CAA has remarkable clinical implications and/or consequences (see Results, Chapter 3.4.2.).

2. ASSOCIATION BETWEEN SSA AND CAA

In a population-based series, the present study (III) statistically proved for the first time that SSA and CAA are not associated with each other. This result has two implications in the amyloid field. Firstly, it opposes the theory on a common predisposing factor to amyloid formation. Secondly, as described in the previous chapter, the common occurrence of these conditions leads to the very common occurrence of one or both of them in the very elderly population. Only a few earlier reports have remotely touched upon the co-occurrence of SSA and CAA, and mostly in animals, not in population-based series. In the report by Yamada (Yamada et al., 1988), the incidence of CAA and SSA are reported separately and without taking notice to the co-occurrence of the conditions. In addition, SSA was detected in only 3% of the participants. In the paper by Uchida (Uchida et al., 1991), CAA was observed in 28 out of 90 elderly dogs, noting that in "some of those having CAA, amyloid deposition was sometimes found in the vascular walls of the heart". Uno (Uno et al., 1996) found CAA in 12 out of 81 monkeys, two of which had amyloid in the pancreas or liver. Unfortunately, cardiac amyloid was not mentioned.

3. CLINICAL, PATHOLOGICAL, AND GENETIC FACTORS IN SSA, CAA, AND AGEL AMYLOIDOSIS

3.1. Age, SSA, and CAA

Although SSA and CAA were not associated (III) with one another, age represents a common denominator between these two conditions. In study I, the prevalence of SSA was not associated with age at death although the frequency of SSA clearly increased along with increasing age at the time of death of the subjects. Unfortunately, in that study, the prevalence numbers concerning different age groups do not represent the true prevalence for SSA, because the individuals who die at a certain age do not represent the whole population at that age. Concerning age and CAA, an association between these two parameters was not studied, as the population in the CAA study was small and the subjects were 95 years of age or more.

An interesting finding was the detection of association between the severity of SSA and age at the time of death. Adding this to the high frequency of both SSA and CAA, the finding raises the question whether amyloid formation

could represent an event associated with normal aging. Some evidence that may support this view include: the detection of amyloid plaques in the brains of non-demented subjects, CAA in leptomeningeal blood vessels in half of normal elderly individuals (Wu et al., 1992), and AMed amyloidosis in all subjects aged 60 years or more.. In addition, concerning TTR, certain biophysical conditions that destabilize the tetramer such as lowered pH have been noticed to accentuate the polymerization of certain mutants, but may even transform wildtype TTR protein into amyloid fibrils under laboratory conditions (McCutchen et al., 1993; McCutchen et al., 1995). Analogously, in living organisms, hypoxic conditions following decreased blood flow due to coronary atherosclerosis or cardiac insufficiency (common conditions in aged individuals), may lower environmental pH in the heart, thus favoring TTR-based amyloid formation. In addition, some of the hereditary amyloid disorders manifest clinically at middle or even old age, although the genetic defect has been present since birth, indicating an association of the clinical disease with accumulation of amyloid during time. This is clearly seen in TTR-related FAP, where orthotopic liver transplantation, by eliminating the source of the amyloidogenic TTR molecule, stops the progression of the disease (Herlenius et al., 2004). CAA is also suggested to be a consequence of decreased Aβ elimination from brain tissue (Feurer and Weller, 1991; McLean et al., 1999) that becomes evident only with the progression of time.

3.2. Gender, SSA, and CAA

The severity of SSA (I) associated with male gender, although the overall frequency of SSA did not significantly differ between genders. In previous studies, SSA has been observed more often in men than in women (Johansson and Westermark, 1991; Kyle et al., 1996; Ng et al., 2005). Presumably, these hospital-based studies have suffered from selection bias, because more severe myocardial amyloidosis in men is more likely to be detected than mild disease in women. On the other hand, the relatively small number of men in the present study (I) may also have affected the results.

As in SSA, the overall frequency of CAA was not associated with gender either. This is in line with the previous population-based study (Masuda et al., 1988) in which the frequency of CAA was relatively equal in men and women aged 90 years or over (42.8 % and 45.8 %, respectively). In the present study, the severity of CAA associated significantly with male gender, similiarly to severity of SSA. In the previous study by Masuda et al., the association between gender and severity of CAA was not mentioned in particular.

3.3. Health-associated risk factors, SSA, and CAA

No previous studies on risk factors for SSA have been available. Although no significant associations were found in study (I), a trend was seen between low BMI and SSA. This trend may be relevant, since diabetes which is not associated with SSA, is associated with obesity or high BMI. However, there are difficulties in interpreting the results with BMI because of its lack of discriminatory power to differentiate body fat and lean mass (Romero-Corral et al., 2006).

In line with a previous study (Yamada et al., 1987), CAA was not associated with HT, smoking, or BMI. However, there was a negative association between DM and CAA, with DM seemingly protecting against CAA. The finding is somewhat unexpected as DM, especially type II DM, has been noted to be a risk factor for dementia (Sima and Li, 2006) which is, in part, strongly associated with CAA (see Results, Chapter 6.2.). The explanation is most probably due to the overall small number of participants in the CAA study, and especially to the small number of diabetics in this age cohort, presumably due to the decreased life expectancy of diabetics (Franco et al., 2007).

3.4. Dementia, SSA, and CAA

No previous reports have been available on the occurrence of dementia in patients with SSA. The present study (III) revealed that clinical dementia is not associated with SSA. In both SSA+ and SSA- groups (see Results, Chapter 3.4.1.), the same number of individuals (11) had normal cognition although the number of individuals in the SSA- group was clearly larger, which raised the question of whether there may be an association between SSA and preserved cognition. However, this tendency was not statistically significant (p=0.09 for all, 0.07 for women).

The present study (II) revealed a significant association between clinical dementia and CAA, consistent with previous studies (Neuropathology Group. Medical Research Council Cognitive Function and Aging Study, 2001; Pfeifer et al., 2002). In the present very elderly Finnish population, clinical dementia was strongly associated with CAA, and this association was even independent of APOE. The APOE ε4 allele also represents a risk factor for neuropil-associated Aβ deposition, including cortical capillary Aβ (Thal et al., 2002). As the APOE ε4 allele is strongly associated with clinical dementia, a tempting question was raised of whether capillary CAA in particular was associated with dementia in this population as well. However, this question remains unanswered here, for several reasons mentioned previously (pp. 74-75) since the presence and severity of CAA was studied using the method by Ellis (Ellis et al., 1996, **Table 8b**, p.52) that does not divide CAA into capillary and non-capillary forms.

Here, the association between clinical dementia and CAA was independent of neuropathological AD. In study II, the modified criteria for neuropathological AD were used. The basis for this was that if the original grading system would have been applied, there would have been a proportion of individuals who could not have been categorized at all, because there is no true linkage between the density of neuritic plaques and the Braak staging system (Geddes et al 1997, Knopman et al., 2003). As a consequence, the number of individuals who potentially could have been included in the study, would have been even smaller. On the other hand, the modified system may have yielded a greater proportion of individuals with neuropathological AD compared to if the original criteria had been used. However, the association between CAA and neuropathological AD in study II was so strong (p=0.0005) that it is very unlikely that using the original criteria would have changed the result to non-significance. In study III, the neuropathological findings were classified using the original CERAD and Braak classifications.

Dementia is the leading cause for the institutional care of elderly individuals in Western countries (Macdonald and Cooper, 2007) including Finland (Nihtila et al., 2007), and it creates a substantial economical burden to the society, e.g. more than 60 billion dollars annually in the USA (Gorshow, 2007). Because of this, CAA is worth more attention, especially as it may present a potential target for medical therapy for clinical dementia. The interrelationships between dementia, CAA, and *APOE* are discussed in detail in the Discussion Chapter 6.2.

3.5. Clinical, laboratory, and radiological findings in AGel amyloidosis

Typical to AGel amyloidosis, the patient in study IV was not demented. He had severe PNP with prominent position and vibration sense losses, areflexia, pseudoathetosis, and ataxia. AGel amyloidosis patients may have mild peripheral neuropathy (Kiuru, 1998), but loss of stance and gait has been rare (Conceicao et al., 2003; Klaus et al., 1959; Kiuru, 1992). Severe ataxia has been previously reported only in one Portuguese patient who did not show any clinical or electrophysiological signs of peripheral neuropathy (Conceicao et al., 2003). Mild ataxia has been shown in Dutch, North American, and Finnish patients (Darras et al., 1986; Kiuru et al., 1999; Kiuru, 1992; Winkelmann et al., 1985), and severe non-cerebellar ataxia in a Czech patient (Klaus et al., 1959) suggests that severe ataxia can also represent a manifestation of G654T-related (de la Chapelle et al., 1992) AGel amyloidosis. The present study (IV) clearly showed that advanced AGel amyloidosis clinically manifests with severe ataxia.

MRI studies in the patient revealed atrophy of the spinal cord, which

can indicate involvement of the posterior columns. In the brain, minor abnormalities related to age rather than to cerebral AGel angiopathy were seen (Kiuru et al., 1999). The advantage of skeletal muscle MRI in amyloid diagnostics has previously been demonstrated in AL amyloidosis (Comesana et al., 1995; Hull et al., 2001; Metzler et al., 1992). In this patient, skeletal proximal leg and back muscle atrophy and fatty degeneration were shown indicating that MRI studies are helpful in amyloid diagnostics. The electrophysiological findings of– decreased or absent sensory potentials and absent SEP responses resemble the ataxic neuropathies (Dalakas and Quarles, 1996). Aberrant central sensory conduction, described in AGel amyloidosis previously (Conceicao et al., 2003; Kiuru and Seppalainen, 1994), may reflect degeneration of central sensory projections, a common phenomenon in sensory ataxia (Lauria et al., 2000). In addition, the electrophysiological studies were consistent with severe chronic sensorimotor neuropathy, mainly with axonal nerve involvement, confirming previous electrophysiological (Kiuru et al., 1999) and sural nerve morphometric studies (Kiuru-Enari et al., 2002). QST may be helpful in differentiating AGel neuropathy from variant TTR-, APOA1-, and AL-amyloid-associated neuropathies with preferential pain and sensory sense losses (Koike et al., 2004).

3.6. General autopsy findings in SSA and CAA

The Chi-squared test showed a trend for association between MIs and SSA, analogous with a previous hospital-based study (Cornwell et al., 1983). The present study confirmed this finding by showing significant association between these parameters in logistic regression analysis after standardizing for confounding factors, which may be inversely associated with SSA. Increased heart weight at the autopsy, a common observation in various myocardial diseases, was also associated with SSA. Here, even heart weight and MIs associated with each other very strongly, with a p-value of 0.000. Although it is not possible to prove causality by using statistical methods, a possible explanation may be that the MIs result in increasing heart weight in the acute phase of infarction due to tissue reactions following hypoxic injury, and also later in the form of scar tissue and reactive hypertrophy of the heart muscle cells. The close association between MIs and SSA leads to a tempting hypothesis that, analogous with CAA, SSA could cause small infarctions due to obliteration of the lumen of the peripheral vessels. However, in order to prove this, it would be advisable to examine larger specimens, including coronary arteries or their branches as well as the smallest peripheral vessels, combined with IHC to visualize the blood vessels.

The association between increased heart weight and lack of CAA (see Results, Chapter 3.6.2.) is in line with the lack of association between SSA and CAA (III).

3.7. Neuropathology in SSA, CAA, and AGel amyloidosis

3.7.1. Brain pathology in SSA, CAA, and AGel amyloidosis

Although TTR-related amyloid has previously been detected in almost every organ of the human body, it was not found in the brain (Pitkänen et al 1984). Thus, SSA was not expected to be associated with, or cause, any brain events. Indeed, no statistical association was noted between SSA and CAA, neuropathological AD, or clinical dementia (III).

Small infarctions and large lobar hemorrhages have been regarded as typical findings associated with CAA (Mandybur, 1986; Vinters, 1987; Olichney et al., 2000). In the present study (II), small infarctions were frequently encountered, representing an essential feature of CAA in this sample of very old individuals. Small cerebral cortical infarctions may be assumed to be caused by amyloid deposition in the arterial wall, followed by thickening and subsequent obliteration of the lumen. Large lobar infarctions, however, were not encountered in the present study (II) in CAA patients. The exact mechanism of hemorrhage in CAA is not known. One explanation, proposed by Maeda *et al.* after performing a computer-assisted three-dimensional image analysis, was that vascular amyloid deposition causes degeneration and loss of smooth muscle cells of media, followed by microaneurysmal dilatation, fibrinoid necrosis, and rupture of the vessel walls (Maeda et al., 1993). This view was supported by the findings of Winkler *et al*. in their mouse model of Aβ-related CAA, in which amyloid deposition was accompanied by an extensive loss of medial smooth muscle cells (Winkler et al., 2001). The analysis of living canine leptomeninges has also shown that Aβ amyloid deposition is associated with loss of vessel wall viability (Prior et al., 1996). Furthermore, small deposits of amyloid fibrils or protofibrils have been reported to exert toxic effects on cells in their vicinity and induce cell death (Lorenzo et al., 1994; Schubert et al., 1995; Janson et al., 1999). Thus, the present study (II) indicates that lobar hemorrhages associated with CAA preferentially occur before age 95, or even 85, as noted even previously. Because such hemorrhages often are lethal, they do not represent a major medical problem in the very elderly. The present study (II) also showed a significant association between CAA and neuropathological AD, in line with a previous study (Pfeifer et al., 2002). AD has been proposed to be a consequence of hypoperfusion of the brain (de la Torre, 1994). Thus, in addition to senile plaques and neurofibrillar tangles, which have traditionally been considered as neuropathological hallmarks of AD, it can be speculated that CAA may also, at least partly, share the same origin. In addition, it possible that the perfusion of the brain is somehow related with drainage and the drainage theory of CAA origin by Weller et al (Weller et al., 1998).

The cerebral findings in the patient with advanced AGel amyloidosis were such as could be expected due to his clinical status: minor amount of

variant Gelsolin-based amyloid in the cerebral blood vessels, noted even previously (Kiuru et al., 1999), and sparse diffuse (non-amyloid) plaques in the cerebral cortex, consistent with normal aging.

3.7.2. Pathology of the spinal cord and peripheral nerves in AGel Amyloidosis and SSA

The clinical findings in the patient with advanced AGel amyloidosis indicated sensory ataxia. Neuropathological studies showed amyloid deposition and atrophy in several levels of the proprioceptive pathway, consistent with earlier pathological reports (Kiuru et al., 1999; Kiuru-Enari et al., 2002). SMI311, an antibody cocktail which stains myelinated nerve fibers, proved to be helpful in showing atrophy in histological samples of the nervous structures. Apart from revealing severe degeneration in the posterior columns of the spinal cord, noted even earlier (Kiuru et al., 1999), this staining showed for the first time the substantial degeneration in the nucleus of the XIIth cranial nerve. This finding in part explains the typical manifestation of the disease: swallowing difficulty. Another cause for this symptom may be enlargement of the tongue, secondary to muscle atrophy and amyloid accumulation. The main differential diagnostically relevant disorders for posterior column, sensory root and/or nerve involvement include tabes dorsalis and diabetic pseudotabes, which were excluded in this patient. Here, severe ataxia led the patient to bed rest and finally resulted in peripheral venous thrombosis, pulmonary embolism, and death. In addition to describing severe ataxia in association with AGel amyloidosis, this case report (IV) also shows that AGel amyloidosis can be fatal.

In SSA, TTR-based amyloid is largely distributed within the organs but no published reports exist on PNP and/or ataxia in the disease (Cornwell et al., 1995). However, the question whether wildtype TTR can cause PNP is clinically relevant regarding several forms of hereditary TTR amyloidosis, especially the most common hereditary TTR amyloidosis in Scandinavia, which is caused by the Val30Met mutation and is the PNP form that causes major disturbance to the patients. Unfortunately, histological samples from spinal cord, spinal nerve roots, or peripheral nerves were not included in the present study setting and thus were not available for neuropathological investigation.

Aβ is normally expressed in the dorsal columns and in the peripheral nerves in both individuals with and without AD (Arai et al., 1991). In the most frequent of the age-associated muscle diseases, inclusion body myositis, Aβ is a typical constituent (Askanas et al., 1992). The background of instabililty of gait, a quite common finding in AD patients, is due rather to anoxic lesions and small infarctions in the brain tissue that are caused by CAA.

3.9. Neuroinflammation

In the present study (II), special interest was focused on inflammation in CAA, and study IV also included an investigation of complement proteins in AGel amyloidosis. The finding of only a few leukocytes around the amyloidotic blood vessels in CAA is in line with earlier reports (Yamada et al., 1996; Eng et al., 2004). During the last 15 years, numerous inflammatory mediators have been observed in AD brains (Akiyama et al., 2000), suggesting that AD may be considered a representative of an inflammatory disorder. Interestingly, the chronic inflammatory response in AD is thought to be locally induced by Aβ without any apparent influx of leukocytes from the circulation (Eikelenboom et al., 2000; Rogers et al., 1992). Indeed, no substantial amount of leukocytes was observed histologically, neither around the cerebral cortical senile plaques nor in any of the other amyloids studied here (SSA and AGel amyloidosis). Complement proteins were present in both amyloid types in which they were studied. Thus, it may be suggested that in amyloid deposits, other factors than leukocytes from the circulation may be able to activate complement. The particular role of complement in CAA and AGel amyloids is discussed later (see Discussion, Chapter 5).

4. GENETIC POLYMORPHISMS, SSA, AND CAA

4.1. *TTR* gene polymorphisms

Apart from the gene for *TTR*, there are no previous population-based reports on potential genetic risk factors in SSA. In the only published genetic study on SSA (Gustavsson et al., 1995), the coding sequence for *TTR* in two patients was sequenced without finding any mutations. This study was extended by performing a similar analysis on six individuals with severe (grade 3) SSA. Five of the individuals studied had the predicted wildtype sequence, but one carried a nonsynonymous rs1800458 SNP polymorphism on exon 2 of the *TTR* gene. This SNP leads to the replacement of glycine to serine in TTR (Gly26Ser mutation), which is a common non-amyloidogenic polymorphism in Caucasian populations (Jacobson et al., 1995). In the whole study population (study I; n=256), the allele frequency was 0.11, which is even somewhat more than reported previously. There was no difference in the allele frequencies among subjects with or without SSA. Thus, the finding indicates that genetic variation in the *TTR* gene is an unlikely explanation for the development of SSA.

4.2. *α2M* polymorphisms

The candidate gene screen in the present study (I) showed a significant association between SSA and exon 24 polymorphism in the *α2M* gene in our study population. α2M is a pan proteinase inhibitor found in the senile plaques of AD (Rebeck et al., 1995), in the tissue deposits of AA (inflammation-associated, formerly referred to as secondary amyloidosis) (Muller et al., 2000), and in the affected tissues in β2-microglobulin (dialysis)-associated amyloidosis (Campistol et al., 1992). The gene for *α2M* is located on chromosome 12. A genetic association between AD and a polymorphic site upstream from exon 18 (Blacker et al., 1998), and the G/G (Liao et al., 1998) and A/A (Myllykangas et al., 1999; Pirskanen et al., 2001) genotypes in exon 24 of the *α2M* gene have been reported. Interestingly, the G/G genotype in exon 24 of *α2M* associated with SSA in this study, whereas the genotype repeatedly reported to associate with AD in the Finnish population has been A/A (Myllykangas et al., 1999; Pirskanen et al., 2001), indicating opposite risk factors for SSA and AD. This finding also supports the observed lack of association between SSA and AD (III). In the cardiac amyloid deposits, α2M protein was not detected. The finding is not conclusive, since IHC on a paraffin-embedded tissue may not be able to detect small amounts of proteins. However, α2M was found in the myocardial scar tissue, which may be due to the activation of extracellular matrix-degrading enzymes after MI (Creemers et al., 2001).

In addition, we did not find any association between the *α2M* gene and CAA. In two previous studies, the A/A polymorphism in exon 24 of the *α2M* gene was associated with AD (Myllykangas et al., 1999: Pirskanen et al., 2001). Thus, association between CAA and the A/A polymorphism in exon 24 was predicted. The lack of association may in part be due to the quite small CAA study population. On the other hand, in a previous study (Yamada et al., 1999a), no association was detected between CAA and an exon 18 polymorphism in the *α2M* gene.

4.3. *Tau* gene polymorphisms

In the present study (I), the candidate gene screen showed a significant association between SSA and two polymorphisms for the *tau* gene. The *tau* gene on chromosome 17 is covered by two extended haplotypes in exon 9, called H1 and H2, where H1 is the more common haplotype (Baker et al., 1999). Yet, the region contains a large number of genes that are in linkage disequilibrium with *tau* H1/H2 (Goedert and Jakes, 2005).

Mutations of the *tau* gene are associated with several neurodegenerative diseases (Goedert and Jakes, 2005) including AD. During the years, views on the significance of *tau* in AD pathogenesis have fluctuated. Studies on association

between *tau* gene polymorphisms and AD have resulted in equivocal results. The association of the *tau* gene with AD pathogenesis is under debate, with some studies showing evidence for association (Myers et al., 2005; Goedert and Jakes, 2005) and others, including a meta-analysis, showing evidence against (Baker et al., 2000; Bullido et al., 2000; Russ et al., 2001; Mukherjee et al., 2007) an association. Explanations for the conflicting findings may lie in the differences between the populations studied, and secondly, that the *tau* gene region is covered by a large number of genes (Goedert and Jakes, 2005) which are in linkage disequilibrium with *tau* H1/H2. Interestingly, before the present finding of association between SSA and *tau, tau* haplotypes or mutations have not been associated with any non-neurological disorder. No previous reports exist on genetic association between the *tau* gene and CAA, and in the present study we did not find any association .

4.4. *APOE* genotypes

Significant association was detected between CAA and the ε4 allele of *APOE*. The association between the severity of CAA and the *APOE* ε4 allele (Polvikoski et al., 1995a), as well as the number of *APOE* ε4 alleles and severity of CAA, has been noted even earlier in slightly younger patients (Chalmers et al., 2003). The association between large CAA-related hemorrhages and *APOE* ε2, reported by McCarron and Nicoll (McCarron and Nicoll, 1998), was not found here.

SSA was not associated with *APOE* polymorphisms, a finding that is in line with lack of association between SSA, CAA, dementia, and AD (III).

4.5. *ACE*, *LPL*, *LRP*, and *Prion protein* polymorphisms

With the exception of *α2M*, none of the other genetic polymorphisms (i.e. the lipid metabolism-, cerebral infarction-, or MI-associated polymorphisms) which in previous studies on the same study population associated with AD, were associated with SSA. These findings suggest that the pathogenic pathways leading to SSA and the above mentioned disorders are unrelated. Moreover, the SSA and AD pathologies were not associated with each other in the Vantaa study material (III).

In study II, in which the "modified criteria" for AD (Polvikoski et al., 2001) were used, both clinical and neuropathological AD were associated with CAA. Thus, since *α2M* previously was found to associate with AD (Myllykangas et al., 1999), the lack of association between CAA and *α2M* was somewhat unexpected. However, the CAA study population had specific features, including lack of association between dementia and the *APOE* ε 4 allele, noted even earlier in the Vantaa population (Juva et al., 2000).

5. IHC FINDINGS IN THE AMYLOID–ASSOCIATED AND OTHER PROTEINS

In all amyloids, the IHC showed slightly more wide-ranged positivity compared to Congo red. The finding is consistent with the finding in mice that TTR amyloid protein has been detected temporally before the amyloid fibrils (Teng et al., 2001). The finding of TTR positivity in a few of the Congo red- negative (control) myocardial samples may reflect the same idea.

Previously, complement proteins have been detected as associative proteins in amyloid deposits in hereditary cerebrovascular ABri and ADan amyloidosis (Rostagno et al., 2002). In the present study (II), the co-localized complement proteins were highly associated with CAA, and furthermore, the amount of complement protein in the amyloidotic blood vessel walls increased with the severity of CAA. The complement proteins studied here included the terminal component C9, suggesting that complement was activated using the classical and alternate pathways as described in the chromosome 13 dementias (Rostagno et al., 2002).Here, Aβ and the complement proteins C3d and C9 were co-localized with amyloid deposits in the amyloidotic blood vessel walls (II). The possibility of nonspecific adherence between these proteins exists, but is minor as both Aβ and complement perform important functions in the brain tissue. The issue of complement, AD, CAA, and Aβ elimination is discussed in detail in the Discussion (Chapter 6.4).

AGel deposition is known to be accompanied by ApoE (Soto et al., 1995), antichymotrypsin (Kiuru et al., 1999), amyloid P component (Kiuru-Enari et al., 2005), and heparin derivatives (Suk et al., 2006). In the present study (IV), complement proteins co-localized with variant gelsolin in the degenerated nerve structures of the patient with AGel amyloidosis. In hereditary TTR amyloidosis, genetic variants of complement 3 and 4 (C3 and C4) have been suggested to guide phenotypic diversity (Nylander et al., 1990). The role of complement proteins in AGel amyloidosis-associated neurodegeneration is not clear. They may represent a reaction in the tissue towards the abnormal variant gelsolin protein because in the degenerated posterior columns, where no amyloid deposition was noted, complement was also not detectable.

Genetic variation in *α2M* is strongly and independently associated with SSA (see Results, Chapter 4.1.). Thus, the protein itself may accompany amyloid material in the myocardial tissue in SSA, although α2M protein was not found in the myocardial amyloid deposits. However, this finding is not conclusive because small amounts of protein may not be detectable in paraffin-embedded tissue material. In contrast, α2M protein was found in myocardial scar tissue. This might be explained by activation of the extracellular matrix-degrading enzymes after MI (Creemers et al., 2001). α2M protein is a constituent of senile plaques in AD (Rebeck et al., 1995), but the presence of this protein within CAA deposits was not specifically studied here.

Phosporylated tau is the constituent of the neurofibrillar tangles that, in addition to senile plaques, represents a pathological hallmark for AD. Due to the strong genetic association between SSA and *tau* (Results, Chapter 4.1.) in the present study (I), the myocardial samples of 14 individuals with moderate or severe SSA were immunohistochemically stained with anti-tau and anti-phosphorylated tau. The reaction was negative within amyloid deposits as well as around them, in the scar tissue and muscle fibers. As with α2M, this finding may be not conclusive, because IHC on paraffin-embedded tisssue may not detect small amounts of protein. It is worth noting that apart from brain tissue in neurodegenerative disorders, phosphorylated tau protein has previously been found in muscle tissue in inclusion body myositis (Askanas et al., 1994).

Here, the occurrence of AMed amyloidosis in intracranial blood vessels was immunohistochemically studied. The lactadherin fragments in the leptomeningeal small and middle-sized blood vessels did not co-localize with the Aβ–related amyloid deposits, but were located in the blood vessel walls outside of the amyloid deposits. The lack of co-localization was confirmed by performing a double staining with Congo red and lactadherin fragments on a case with advanced CAA. Indeed, in all types of amyloid studied until now, only one amyloid fibril protein has been encountered.

6. EFFECTS OF MULTIPLE FACTORS ON SSA AND CAA

6.1. Multivariate analysis of age, *α2M* and *tau* gene polymorphisms, and MIs on SSA

The Chi-squared test showed a trend for association between MIs and SSA (see Results, Chapter 3.6.1.), analogous with a previous hospital-based study (Cornwell et al., 1983). After standardizing for confounding factors, the logistic regression analysis revealed an independent association between these two conditions. Increased HW at autopsy, a common observation in various myocardial diseases, was also associated with SSA (see Results, Chapter 3.6.1.). Thus, the findings indicate that SSA is often combined with other myocardial pathologies, which may be one of the reasons why SSA is so seldom clinically diagnosed.

6.2. Dementia, APOE, and CAA

CAA was associated with clinical dementia (see Results, Chapter 3.4.2.), and *APOE* ε4 (see Results, Chapter 4.2.). However, clinical dementia was not associated, within the power limits of the study, with the presence of *ApoE* ε4 in this study population (see Results, Chapter 6.2.; Juva et al., 2000), in contrast with several other studies. This lack of association may be a power problem, since a trend towards increased severity of CAA and increased frequency of ε4 was seen in the demented subjects compared to nondemented subjects. Remarkably, the demented group included not only the AD subjects but also vascular and other dementias as well, and CAA was associated with dementia in both groups with and without NIA-RI AD (Results, Chapter 6.2.). All of these results suggest that CAA is not a secondary phenomenon associated with AD, but is independently associated with clinical dementia in this age group. This finding, combined with the remarkable obliteration of the affected vessels in CAA in the histological samples, clearly supports the prevailing theory on the origin of AD. This "amyloid cascade hypothesis" by Hardy and Selkoe suggests that accumulation of amyloid β-peptide in the brain is the key event in the development of AD, but that the rest of the disease process including formation of neurofibrillar tangles containing tau protein, results from imbalance between the production and clearance of Aβ (Hardy and Allsop, 1991; Hardy and Selkoe, 2002). However, even in the 1990s, Jack de la Torre brought out the animal model-based theory, that dementia is caused by impaired brain perfusion (de la Torre and Fortin, 1994; de la Torre, 1997; de la Torre and Stefano, 2000). The possibility of a primary role of the CNS vascular system in AD pathogenesis in humans is also supported by Zlokovic *et al*., who suggested that alterations in the transport across the BBB may have a substantial role in the development of cerebral β-amyloidosis in AD (Zlokovic, 2002; Zlokovic, 2004). It has even been suggested that the soluble forms of Aβ may be in response of many of its neurotoxic and vasculotoxic effects, thus representing a potential link between AD and vascular mechanisms of dementia (Zlokovic, 2005). Dermaut has suggested that CAA may represent a pathogenic lesion with significant contribution to progressive neurodegeneration in AD (Dermaut et al., 2001), based on investigations of a family with an early-onset novel presenilin-1 mutation (L282V)-associated AD. A scheme for AD pathogenesis in which ischemia plays a crucial role in influencing and linking β-amyloid deposition to neuronal damage and clinical disease has recently been presented by Pluta (Pluta, 2004). Other pathogenic pathways for AD development include oxidative stress and mitogenic dysregulation, which are even thought to cause other neurodegenerative diseases (Zhu et al., 2007) and mitochondrial dysfunction (Chen et al., 2006).

6.3. Clinical dementia, CAA, and SSA

CAA associated with clinical dementia in both the SSA+ and SSA- groups (see Results, Chapter 6.3.). This finding further strengthens the independency of association between CAA and clinical dementia, an association that is also independent of SSA.

6.4. Complement, AD, CAA, and Aβ elimination

In AD, complement is a part of the inflammatory cascade in senile plaques, triggered by Aβ as described earlier (Akiyama et al., 2000). Other complement-activating factors in the brain include infarction (Lindsberg et al., 1996), hemorrhage, infection, and possibly even a sustained breach in the BBB (Bradt et al., 1998). In the present study, acute or old ischemic lesions or hemorrhages were encountered only in a subset of cases, suggesting that complement activation in CAA is probably not explained by hypoxic stress. In fact, cases with small cortical infarcts showed somewhat less complement activity, perhaps due to increasing tissue clearance associated with scarring of the infarction. The study also revealed a lack of association between iron and complement deposition, suggesting that complement-mediated lysis of cell- or basal membranes was not present or did not cause ongoing blood extravasation. Both of these findings suggest that in these very old subjects neither ischemia nor blood extravasation activates complement. Thus, Aβ should be considered as a respectable candidate as a trigger of complement activation in these very elderly individuals. Apart from being a part of the inflammatory reactions, complement also has a role in assisting with the removal of targeted molecules (Rogers et al., 2002). Thus, complement might be associated with failure in eliminating Aβ, which is proposed to result in CAA (Feurer and Weller, 1991; McLean et al., 1999). *In vitro* experiments show that Aβ 1-40 is cleared from young murine brains via the endothelium into the blood, mediated by low density lipoprotein (LDL) receptor related protein–1 (LRP-1), α_2-macroglobulin, and possibly also ApoE (Ghersi-Egea et al., 1996; Shibata et al., 2000). A clearance by this route directly into the blood is reduced in older mice, in whom perivascular pathways seem to be more significant (Shibata et al., 2000). Recently, the perivascular route of Aβ elimination from the extracellular spaces of the human brain along the walls of capillaries, as well as cortical and leptomeningeal arteries, has recently been defined (Preston et al., 2003).

It has been shown that Aβ is able to activate complement by binding to C1Q in an antibody-independent fashion (Rogers et al., 2002; Bradt et al., 1998). Although C1Q was not investigated in the present study (II), co-localization of complement proteins (C3d and C9) with amyloidotic blood vessels in CAA may be representative of this binding. Interestingly, only the

smallest vascular amyloid deposits were devoid of complement proteins. This may imply that there must be sufficient Aβ to activate complement. However, the involvement of complement proteins in the complex course of Aβ elimination and subsequent development of CAA requires further investigation.

7. THE STRENGTHS AND LIMITATIONS OF THE STUDY

The strength of the Vantaa 85+ study is that it is prospective and population-based, thus lacking selection bias often involved in epidemiological studies. The study is powerful because the Finnish population is based on a small number of founders, and therefore the number of genetic risk factors for diseases may be smaller than in other populations. The autopsy rate of the Vantaa 85+ study, 51%, is the second highest among all population-based neuropathological autopsy studies with only the Japanese Hisayma study exeeding this rate (Zaccai et al., 2006). Although the autopsy frequencies in both the SSA and CAA studies were somewhat lower, the results are reliable, especially as the diagnosis of both SSA and CAA cannot be made without a histological sample.

On the other hand, due to the special nature of the Finnish population, the results may thus be limited to this population only. In addition, the number of subjects in the study population is relatively small, and thus the possibility of false positive results cannot be totally excluded, albeit the population was very carefully characterized. Finally, the utility of SNP-based association studies for complex human diseases has become an increasingly debated question (Fallin et al., 2001). In the future, ultramodern techniques using high-density whole-genome association approaches (Coon et al., 2007) may prove to be more informative in studies on multigenic diseases.

CONCLUSION

This population-based study shows that SSA and CAA are not associated with each other, but either or both of them are detected in two-thirds of very elderly individuals. Old age, myocardial infarctions, exon 24 polymorphisms of the α-2M gene, and the H1/H2 polymorphism of the *tau* gene is associated with SSA, while clinical dementia and *APOE ε4* genotype is associated with CAA. A strong association between CAA and clinical dementia was demonstrated, which was independent of *APOE ε4* genotype and neuropathological AD. This highlights the clinical significance of CAA in very elderly individuals, in whom serious end-stage complications of CAA (e.g. multiple infarctions and hemorrhages) are seldom. Further, complement 3d and 9 were present in amyloid deposits in CAA and AGel amyloidosis, and advanced AGel amyloidosis was shown to be fatal. Due to the high prevalence of SSA in the elderly population, prospective clinical studies are needed to define its clinical significance.

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