Clinical and experimental studies of hereditary and acquired forms of amyloidosis

DECLARATION

This thesis has been composed entirely by myself unless otherwise stated. The work contained in the thesis has not been submitted in candidature for any other degree, diploma, or professional qualification.

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Abstract

Although the proteinaceous nature of amyloid deposits was first recognised more than 100 years ago, characterisation of amyloid fibril precursor proteins was made possible only recently following the development of a method of amyloid fibril protein extraction. Since then, 15 different amyloid proteins have been identified. In this thesis the development of a novel "mini-fibril" extraction technique, allowing for the first time the isolation of amyloid fibril proteins from tiny milligram quantities of biopsy tissue, is described. This led directly to the identification of a novel amyloid fibril precursor protein in a case of primary localised orbital amyloidosis and enabled the identification and full characterisation of a novel apolipoprotein AI variant in an Australian family with hereditary renal amyloidosis (HRA). In a large Spanish family with HRA, a novel apolipoprotein AI deletion variant was identified as the precursor protein. In both these families, simple dsDNA sequencing confirmed complete concordance between mutation and the exclusive presence of variant protein sequence in the fibril proteins. Two other families with previously described apolipoprotein AI and fibrinogen α-chain variants, together with an Italian family and a Colombian family with novel transthyretin variants associated with familial amyloid polyneuropathy were also studied. Clinical, biochemical, histological and scintigraphic studies using radio-labelled serum amyloid P (SAP) component, an *in vivo* technique for the diagnosis of systemic amyloidosis provided unique information on the pattern of distribution of amyloid and the natural history of the disease in addition to demonstrating the virtually complete penetrance of these autosomal dominantly inherited mutations.

Dialysis-related amyloid (DRA) is an invariable complication of long term haemodialysis (HD). The long term outcome of successful renal transplantation and the prevalence of this disease in patients dialysed predominantly by continuous ambulatory peritoneal dialysis (CAPD) compared to long term HD populations were studied for the first time using radiolabelled SAP scintigraphy and radiology. These studies provide unique evidence for regression of DRA deposits following successful renal transplantation in contrast to relentless progression of DRA in patients maintained on

HD. Intriguingly, prevalence of the disease appears to be comparable in long term CAPD populations, but clinical expression of the disease may be different, suggesting that the modality of HD itself may be important in modulating symptoms associated with the deposits. In both HD and CAPD patients, CTS was the commonest presenting feature of DRA, the wrists were invariably affected scintigraphically, age and duration of dialysis were confirmed as the most important risk factors and the prevalence of DRA was found to be frequently underestimated clinically and radiologically. Histological and scintigraphic studies demonstrated that in both hereditary and acquired forms of amyloidosis, there was an asymptomatic prodromal phase that may last several years.

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Ethical Approval

All individuals who participated in the clinical research studies described in this thesis gave full informed consent in a format approved by the Research Ethics Committee of the Hammersmith Hospital. The dosage and administration of radioactive isotopes were approved by the Administration of Radioactive Substances Advisory Committee of the Department of Health and Social Security.

Abbreviations

AA	amyloid A protein
AEF	amyloid enhancing factor
AL	amyloid light chain
AP	amyloid P component
ApoAI	apolipoprotein AI
APR	acute phase response
β2-M	β2-microglobulin
BSA	bovine serum albumin
CAPD	continuous ambulatory peritoneal dialysis
CCF	congestive cardiac failure
CRF	chronic renal failure
CRP	C-reactive protein
CTD	carpal tunnel decompression
CTS	carpal tunnel syndrome
Da	daltons
DMSO	Dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DRA	dialysis-related amyloid
DW	distilled water
EDTA	ethylene diamine tetra-acetic acid
ESMS	electrospray mass spectrometry
ESRF	end-stage renal failure
FAP	familial amyloid polyneuropathy
g	gravitational force
GAGs	glycosaminoglycans
HD	haemodialysis
HRA	hereditary renal amyloid
HSA	human serum albumin

IEF	isoelectric focussing
iv	intravenous
Ig	immunoglobulin
JCA	juvenile chronic arthritis (Still's disease)
kDa	kilodaltons
MALDI-TOF	matrix assisted laser desorption ionisation time of flight
MBq	megabecquerel
NBS	normal bovine serum
NHS	normal human serum
NMS	normal mouse serum
NS	nephrotic syndrome
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RPMS	Royal Postgraduate Medical School
RRT	renal replacement therapy
R_x	treatment
SAA	serum amyloid A protein
SAP	serum amyloid P component
SDS	sodium dodecyl sulphate
T ½	half-time
TDD	total dialysis duration
TTR	transthyretin
T_x	transplant

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Chapter 1 - Introduction

Amyloidosis

Definition

Amyloidosis is a disorder of protein metabolism characterised by the extracellular deposition of abnormal protein fibrils [1-3]. It may either be localised, or systemically distributed throughout the body (Table 1.1 & Table 1.2), causing organ damage and serious morbidity. Major organ involvement, especially of the kidneys and heart is usually fatal.

Protein fibrils form the bulk of amyloid deposits and are derived from a remarkably heterogeneous variety of precursor proteins. The remaining specific amyloid constituents are largely sulphated GAGs and SAP.

Amyloid is defined by its pathognomonic tinctorial properties with the dye Congo red [4], and by its characteristic ultrastructural morphology. The term "amyloid" (Greek for starch-like) was introduced by Virchow in 1854 on observing that the waxy infiltrated organs of patients who had died with chronic inflammatory diseases such as tuberculosis and osteomyelitis, reacted with iodine in the same manner as starch and cellulose [5]. That these deposits were of a protein nature was demonstrated in 1859 by Fredereich and Kekule and despite this the term "amyloid" was retained [6].

In the past 25 years, much has been learned of the biochemistry and pathogenesis of amyloid, though it has as yet had only a limited impact on therapy. The fibrillar nature of amyloid was first observed in 1959 by Cohen and Calkins, and by Spiro [7]. A major breakthrough came in 1968 when Pras et al described the first satisfactory method for isolating amyloid fibrils from tissue in a sufficiently pure form for chemical analysis [8]. This permitted the precise identification of amyloid fibril proteins which was first achieved for immunoglobulin light chains (AL) by Glenner et al

and subsequently for amyloid A protein (AA), prealbumin and for many other proteins [9].

Classification and Nomenclature

The development of a method of fibril extraction [8] and its recent modification, [10] means that amyloid fibril proteins can now be extracted from tiny milligram quantities of biopsy tissue for full biochemical characterisation. This have led to a new method of classification of amyloid [11] according to the nature of the fibril proteins of which 15 have so far been identified (Table 1.1 & Table 1.2), replacing earlier classifications based on clinico-pathological features. In many cases related or identical proteins, which are clearly the precursors of the fibril proteins, are produced locally or are present in the circulation. In other cases the precursor-product relationship is not yet firmly established, whilst in many types of amyloid the fibril protein remain unidentified.

Clinical Amyloidosis Syndromes

AA (Reactive) Amyloidosis

Diseases complicated by AA amyloidosis are characterised by their ability to provoke a sustained acute phase response and can be divided into 3 main types of chronic disease: chronic inflammatory disorders, chronic local or systemic microbial infections, and malignant neoplasms (Table 1.3). Amongst the connective tissue diseases, systemic lupus erythematosus (SLE) is unique in that it is only very exceptionally complicated by AA amyloidosis and a similar contrast exists between Crohn's disease in which amyloid occurs in 1 to 8% of cases and ulcerative colitis in which it is extremely rare.

Table 1.1 Acquired amyloidosis syndromes

CLINICAL SYNDROME		FIBRIL PROTEIN
Systemic AL amyloidosis, associated with immunocyte dyscrasia, myeloma, monoclonal gammopathy, occult dyscrasia		AL fibrils derived from monoclonal immunoglobulin light chains
Local nodular AL amyloidosis (skin, respiratory tract, urogenital tract, etc) associated with focal immunocyte dyscrasia		AL fibrils derived from monoclonal immunoglobulin light chains
Reactive systemic AA amyloidosis, associated with chronic active diseases		AA fibrils derived from serum amyloid A protein (SAA)
Senile systemic amyloidosis		Transthyretin (TTR) derived from plasma TTR
Focal senile amyloidosis:	atria of the heart	Atrial natriuretic peptide
	brain	β-protein
	joints	Not known
	seminal vesicles	Seminal vesicle exocrine protein
	prostate	β2-microglobulin
Non-familial Alzheimer's disease, Down's syndrome		β-protein derived from β-amyloid protein precursor (APP)
Sporadic cerebral amyloid angiopathy		β-protein derived from β-amyloid precursor protein (APP)
Sporadic Creutzfeldt-Jakob disease, kuru (transmissible spongiform encephalopathies, prion diseases)		Prion protein (PrP) derived from prion protein precursor

Type II diabetes mellitus	Islet amyloid polypeptide (IAPP), amylin, derived from its precursor protein
Endocrine amyloidosis, associated with APUDomas	Peptide hormones or fragments thereof (*e.g.* precalcitonin in medullary carcinoma of thyroid)
Dialysis-related amyloidosis; localised to osteoarticular tissues or systemic	β2-microglobulin derived from high plasma levels
Primary localised cutaneous amyloid (macular, papular)	? Keratin-derived
Ocular amyloid (cornea, conjunctiva)	Not known
Orbital amyloid	AH fibrils derived from immunoglobulin heavy chain in one case (see Chapter 7)

Table 1.2 Hereditary amyloidosis syndromes

CLINICAL SYNDROME	FIBRIL PROTEIN
Predominant peripheral nerve involvement, familial amyloid polyneuropathy (FAP). Autosomal dominant	Transthyretin (TTR) genetic variants (most commonly Met30, but over 40 others described)
Predominant peripheral nerve involvement, familial amyloid polyneuropathy (FAP). Autosomal dominant	Apolipoprotein AI (apoAI) N-terminal fragment of genetic variant Arg26
Predominant cranial nerve involvement with lattice corneal dystrophy. Autosomal dominant	Gelsolin, fragment of genetic variant Asn187 or Tyr187
Hereditary renal (non-neuropathic) amyloid with prominent visceral involvement (Ostertag-type). Autosomal dominant	ApoAI, N-terminal fragment of genetic variants Arg26, Arg50, Arg60, or deletion/insertion variant.
Hereditary renal (non-neuropathic) amyloid with prominent visceral involvement (Ostertag-type). Autosomal dominant	Lysozyme genetic variant Thr56 or His67
Hereditary renal (non-neuropathic) amyloid with prominent visceral involvement (Ostertag-type). Autosomal dominant	Fibrinogen α-chain, fragment of genetic variants, Leu554 or Val526
Predominant cardiac involvement, no clinical neuropathy. Autosomal dominant	TTR genetic variants Thr45, Ala60, Ser84, Met111, Ile122

Hereditary cerebral haemorrhage with amyloidosis (cerebral amyloid angiopathy). Autosomal dominant	
Icelandic type (major asymptomatic systemic amyloid also present)	Cystatin C, fragment of genetic variant Glu68
Dutch type	β-protein derived from genetic variant APP Gln693
Familial Alzheimer's disease	β-protein derived from genetic variant APP Ile717, Phe717 or Gly717
Familial dementia - probable Alzheimer's disease	β-protein derived from genetic variant APP Asn670, Leu671
Familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome (hereditary spongiform encephalopathies, prion diseases)	Prion protein (PrP) derived from genetic variants of PrP precursor protein 51-91 insert, Leu102, Val117, Asn178, Lys200
Familial Mediterranean fever, prominent renal involvement. Autosomal recessive	AA derived from SAA
Muckle-Well's syndrome, nephropathy, deafness, urticaria, limb pain	AA derived from SAA
Cardiomyopathy with persistent atrial standstill	Not known
Cutaneous deposits (bullous, papular, pustulodermal)	Not known

AA amyloid deposition has a predilection for parenchymal organs and may be widely distributed without causing symptoms [12]. The spleen is always affected but the common involvement of the kidneys is most closely associated with an adverse prognosis and it usually presents with non-selective proteinuria due to deposition in the renal glomeruli. This may lead to nephrotic syndrome before terminating in end stage renal failure. The second most common presentation is with organomegaly. Involvement of the heart and gastrointestinal tract is frequent though it rarely causes functional impairment [1, 13].

AL (Monoclonal Immunoglobulin) Amyloidosis

AL amyloid may be associated with almost any dyscrasia of the B lymphocyte lineage ranging from frank malignancy of plasma cells (multiple myeloma) to "benign" monoclonal gammopathy in which the only demonstrable abnormality may be the overproduction of monoclonal light chains. In some cases AL amyloid may be the only evidence of an underlying plasma cell dyscrasia. AL amyloid occurs in up to 15% of cases of myeloma and in a lower proportion of other plasma cell disorders [14, 15].

AL amyloid affects principally the mesenchymal tissues causing peripheral and autonomic neuropathy, carpal tunnel syndrome, macroglossia, restrictive cardiomyopathy and arthropathy of the large joints although visceral organs or indeed any tissue may be involved. It has a poor prognosis especially when the heart is involved (6-18 months) when the usual presentation is of a restrictive cardiomyopathy [16].

Table 1.3 Conditions associated with reactive systemic (AA) amyloidosis

Chronic inflammatory disorders
Rheumatoid arthritis
Juvenile chronic arthritis
Ankylosing spondylitis
Psoriasis and psoriatic arthropathy
Reiter's syndrome
Adult Still's disease
Behçet's syndrome
Crohn's disease
Chronic microbial infections
Leprosy
Tuberculosis
Bronchiectasis
Decubitus ulcers
Chronic pyelonephritis in paraplegics
Osteomyelitis
Whipple's disease
Malignant neoplasms
Hodgkin's disease
Renal carcinoma
Carcinomas of gut, lung, urogenital tract
Basal cell carcinoma
Hairy cell leukaemia

Hereditary systemic amyloidosis

There are many forms of hereditary systemic amyloidosis and most of them are rare. However autosomal dominant inheritance and the adult onset of symptoms have ensured persistence of the trait which causes this devastating disorder. All the various syndromes are characterised by point mutations leading to the production of variant amyloidogenic proteins. Variant transthyretin which causes familial amyloid

polyneuropathy (FAP) was the first of these proteins to be identified [17] and more than 40 mutations in the transthyretin gene have since been described [14, 15]. Mutations in the apolipoprotein AI [18, 19] and gelsolin [20] gene may also be involved and more recently mutations in the lysozyme [21] and α fibrinogen [22] gene leading to expression of amyloidogenic variants in patients with Ostertag-type, non-neuropathic amyloidosis have been discovered.

Familial Mediterranean Fever (FMF) is an autosomal recessive inflammatory disease that may be complicated by AA amyloidosis especially in some ethnic groups.

Senile Systemic Amyloidosis

Some amyloid is present in all autopsies on aged individuals. It may be systemic (usually due to normal wild type transthyretin) with deposits predominantly found in the heart and none in the brain, or it may be focal, involving the brain (β protein), corpora amylacea of the prostate (β2-M), joints and seminal vesicles (precursor protein(s) unknown) [1].

Dialysis related amyloid

This form of amyloid in which fibrils consist of β2–M [23] affects a large proportion of patients receiving long term haemodialysis and has also been reported in patients on CAPD [24] and even in a patient with chronic renal failure but not requiring dialysis [25]. Initially thought to be confined to joints, bones and periarticular tissues deposits of β2–M have now been reported in many other tissues and several cases of fatal extensive disease have been reported. It causes carpal tunnel syndrome, arthralgias of the large joints and bone cysts often leading to pathological fractures. Renal transplantation is the only effective treatment with rapid normalisation of blood β2–M levels and resolution of symptoms [13, 26].

Localised amyloidosis

Deposits of amyloid localised to particular organs or tissues occur in a wide variety of different forms and are presumably consequent on either the local production of fibril precursors (nodular AL amyloid, cutaneous amyloid, endocrine amyloid) or the properties of a particular microenvironment that predisposes to localisation and fibril formation of systemically distributed circulating precursor proteins (e.g. bony/periarticular β2-M in DRA. Many local forms of amyloid are common accompaniments of ageing and rarely of clinical significance. Protein precursors of tumour related amyloid remain unidentified [27] but are probably derived from tumour related or locally produced proteins such as keratin.

Cerebral amyloid

The brain and intracerebral blood vessels are rarely affected in the systemic amyloidoses but are common and important sites for the local deposition of amyloid in the absence of amyloid elsewhere in the body. The most frequent and important type of amyloid in the brain is that related to Alzheimer's disease, which is the commonest cause of dementia. The vast majority of cases of Alzheimer's disease are sporadic but there are also families with an autosomal dominant pattern of inheritance [28, 29]. The triad of cerebral amyloid angiopathy, neurofibrillary tangles and neuritic plaques seen in Alzheimer's disease (AD) is also seen in dementia that universally occurs in Down's syndrome patients over the age of 40 years [30]. These intracerebral and cerebrovascular amyloid deposits are hallmarks of the neuropathological diagnosis and are derived from the amyloidogenic β-protein fragment of the amyloid precursor protein (APP).

Fibril proteins and their precursors

AA

Amyloid A (AA) which has a molecular weight around 8000 daltons is derived from the circulating precursor serum amyloid A (SAA) by proteolytic cleavage. SAA, an apolipoprotein of HDL particles, is an acute phase reactant. Synthesis of SAA by hepatocytes is regulated by cytokines especially IL-1, IL-6 and TNF and its concentration may increase by up to several thousand fold during the acute phase response but its function is not known. SAA is polymorphic but there is no evidence that any particular form is either more or exclusively amyloidogenic.

AL

AL proteins consist of either the whole molecule or fragments of monoclonal immunoglobulin light chains, and in any individual case there may be a mixture of these. The fragments are derived from the N-terminal region and consist of whole or part of the variable (V_L) domain. The L chain of the circulating or urinary monoclonal paraprotein is either identical to, or clearly the precursor of, AL isolated from the amyloid deposits. AL is more commonly derived from λ chains than from κ chains despite the fact that κ chains predominate among normal immunoglobulins and the paraprotein products of immunocyte dyscrasias.

Transthyretin

Transthyretin (TTR), formerly known as prealbumin, is produced by the liver and choroid plexus and is involved in the transport of thyroid hormones and vitamin A. Genetic variants of the TTR molecule, all involving a single amino acid substitution, are the most common cause of hereditary amyloidosis (Table 1.4). TTR which has a

notably high content of beta sheet structure, is an inherently amyloidogenic molecule, and little change is required in its structure for this property to be greatly enhanced.

Table 1.4 Amyloidogenic and non-amyloidogenic variants of transthyretin

10	20	30	40
GPTGTGESKC	PLMVKVLDAV	RGSPAINVAV	HVFRKAADDT
R	***E***	***M***	***I P***
		A	***L***
		L	
S			

50	60	70	80
WEPFASGKTS	ESGELHGLTT	EEEFVEGIYK	VEIDTKSYWK
G T R AR	***P GP HKA***	***K L LHN***	***A Y***
A I	***R***		
V			

90	100	110	120
ALGISPFHEH	AEVVFTANDS	GPRRYTIAAL	LSPYSYSTTA
S QN	***G***	***V***	***M C***
N			
		R T	M
		V	

127
VVTNPKE
I

The wild type sequence of transthyretin is shown above. Amino acid substitutions shown in bold italics below are associated with transthyretin amyloidosis; others shown in normal type are not.

β-protein

The protein in the intracerebral and cerebrovascular amyloid of Alzheimer's disease, Down's syndrome and hereditary amyloid angiopathy of Dutch-type is β

protein, a 39-43 residue sequence derived by proteolysis from APP, a high molecular weight protein encoded on the long arm of chromosome 21. There is controversy over whether or how the β-protein fragment *per se*, or the amyloid fibrils which it forms, contribute to the neuronal dysfunction damage which underlie the dementia. However, the fact that APP mutations may cause Alzheimer's disease [28, 29] and produce the same neuropathology as sporadic cases, including tangles, argues strongly that the APP and β-protein pathway can be of primary pathogenetic significance. Further support for this concept comes from the fact that all individuals with Down's syndrome (trisomy 21) over the age of 40 years develop typical Alzheimer's disease [30]. Since APP is coded by a gene on chromosome 21 it seems that excessive gene dose with wild-type APP can have the same effect as the APP variants in familial Alzheimer's disease.

β2-microglobulin (β2–M)

β2–M is the amyloid fibril protein of dialysis-related amyloidosis [23]. It is a non-polymorphic single chain polypeptide with a molecular weight of 11,800 daltons, and is the light chain of Class I MHC antigens present on all nucleated cells. It is continually shed from cell membranes and is normally filtered by the kidney and catabolised in the proximal tubule. The failure of haemodialysis membranes to clear β2–M efficiently leads to persistently raised plasma levels and this presumably predisposes to its deposition as amyloid fibrils. The strong affinity of β2–M for collagen *in vitro* may explain the striking predisposition for β2–M amyloid to deposit in the collagen rich tissues of the joints [31].

Cystatin C

Cystatin C is an inhibitor of cysteine proteinases and a 110 variant residue of this protein, with an amino acid substitution (glutamine for leucine) at position 58, is the subunit of amyloid fibrils in Icelandic patients with hereditary cerebral haemorrhage

with amyloidosis [32]. These cerebrovascular amyloid deposits lead to haemorrhagic and thrombotic strokes, usually causing death before the age of 40 years.

Polypeptide hormones

Amyloid often occurs in endocrine organs in connection with ageing and in some polypeptide hormone secreting tumours. The localised amyloid deposits occurring in certain polypeptide hormone secreting tissues consist mainly of hormone or pro-hormone. Examples are calcitonin related amyloid in medullary carcinoma of the thyroid, islet amyloid polypeptide derived amyloid which occurs in 95% of patients with Type II diabetes [33] and atrial natriuretic peptide in isolated atrial amyloidosis.

Nature of amyloid deposits

Fibrillar components

Amyloid is defined histologically by its binding of the dye Congo Red and the subsequent green birefringence when viewed under polarised light [4]. This unique property resides in the fibrils themselves and is retained in isolated fibril preparations. Most though not all, proteins which are precursors of amyloid fibrils are rich in β-sheet secondary structure. This, together with the apparent resistance of amyloid fibrils to proteolysis, led to the concept that amyloid fibrils consist exclusively of a stack of anti-parallel β-pleated sheets arranged with their long axis perpendicular to the long axis of the fibril, resembling the structure proposed for silk, which is also highly proteinase resistant. However, fibrillar morphology per se does not require β-pleated sheet structure, and X-ray fibril diffraction studies suggest that a shared repeating structure in different amyloid fibrils may derive from similar intermolecular packing motifs rather than a shared secondary structure.

Another constant feature of all amyloid deposits is the universal presence of sulphated glycosaminoglycans (GAGs) which are probably laid down simultaneously

with the fibrils. These fibril associated GAGs are heparan sulphate and dermatan sulphate in all forms of amyloid which have been investigated [34]. Fibrils isolated by water extraction contain 1-2% by weight of GAG carbohydrate, none of which is covalently associated with the protein. The significance of GAGs remains unclear but there is strong presumptive evidence that they may be the ligands to which SAP, another universal constituent of amyloid deposits, binds.

Non-fibrillar components

Human SAP, a decameric plasma glycoprotein, binds in a calcium dependent manner to all forms of amyloid fibril and is universally present in amyloid deposits [35], including the cerebral amyloid of Alzheimer's disease. SAP which is produced only by the liver is also a normal constituent of glomerular basement membrane and elastic fibre microfibrils.

SAP is remarkably resistant to proteolytic degradation in the presence of calcium. This resistance to proteinase digestion is likely to be an important aspect of the normal function of SAP, and may also contribute to the persistence of amyloid deposits by protecting them against digestion. Protection could result simply from coating of the abnormal amyloid fibrils by SAP, which is completely unaltered with respect to its normal circulating form, and which would therefore not be expected to trigger macrophage activation and phagocytosis. However, the proteinase resistance of SAP itself may be a significant factor.

The three dimensional structure of SAP has recently been solved to atomic resolution [36]. Availability of the complete structure to 2Å resolution of SAP and determination of its ligand binding sites now offer the opportunity of direct modelling of competitive inhibitors of SAP binding and for producing binding site homologues, either of which could be used as drugs to displace SAP from amyloid deposits *in vivo*. This would open up new avenues for treatment of amyloidosis, enabling the body to mobilise and degrade the fibrils which may otherwise be inappropriately protected by SAP.

Pathogenesis

Fibril Formation

A necessary condition for the formation and deposition of amyloid fibrils is the presence of an autologous protein precursor, which is either circulating or produced locally, and which is abnormal either in structure, concentration or both. Until recently, it was generally accepted that the pathogenesis of amyloid fibril deposits involved the partial proteolytic cleavage of qualitatively or quantitatively abnormal precursor proteins into fragments with a propensity for aggregating into anti-parallel beta pleated sheets [2, 3]. There is however now evidence that intact undegraded variant transthyretin [37] and β2–M [38] molecules can aggregate to form fibrils in FAP and DRA respectively.

Although the pathogenesis of amyloid fibril formation is not known, the primary structure of precursor proteins is crucial in determining their amyloidogenicity. The evidence is provided by the genetic variants of transthyretin [39-41], apolipoprotein AI [42-44] and lysozyme [21] in which a single amino acid substitution converts the non-amyloidogenic wild type proteins into highly amyloidogenic variants.

The presence of precursor proteins is obviously necessary for fibril formation but the factors that determine the deposition of precursor proteins as amyloid fibrils in some individuals but not others, and the variation in the time and anatomical distribution of deposits are not known. Mice given repeated inflammatory stimuli over a period of 3 weeks or more eventually develop AA amyloidosis but the latent period can be reduced to 1-2 days in mice which have previously received a single intravenous injection of an extract of amyloidotic tissue. The nature and mode of action of this so called "amyloid enhancing factor" [45] is not known, but may be responsible for the marked individual variation in susceptibility to fibril formation and elucidation of the processes involved will clearly be of considerable clinical relevance.

Fibril Degradation

The natural history of amyloid deposits is to persist and accumulate. This apparent resistance to proteolytic degradation has been ascribed to the silk-like antiparallel β-pleated sheet structure of the native protein and it is also possible that the universal presence of SAP, in an apparently native unaltered state, may be involved in some way.

However, amyloid deposits can and do regress once supply of the precursor proteins is reduced. Evidence for this was previously provided only histologically by isolated case reports [46]. However, there is now collective data from serial studies with ^{123}I-SAP scans demonstrating regression of amyloid deposits following treatment to reduce precursor protein levels in patients with AA, AL β2–M amyloidosis and in FAP due to TTR mutations [12, 47-50].

Diagnosis

Clinical features of amyloidosis are extremely varied and non-specific and it's diagnosis which often requires a high index of suspicion, is confirmed by demonstration of tissue amyloid deposits which when stained with the dye Congo red [4] and viewed under cross polarised light produces characteristic apple-green birefringence. This universal congophilic property of amyloid remains the gold standard by which other diagnostic techniques are judged in amyloidosis. Typing of amyloid fibril protein can usually be determined, most simply, by immunohistochemical staining and this have rendered obsolete previous attempts at classification of amyloid on the basis of differences in Congo red staining following pre-treatment of tissue sections with potassium permanganate, alkaline guanidine or autoclaving [14]. Finally, amyloid fibrils may be demonstrated directly by electron microscopy of tissue sections but a negative result does not exclude the diagnosis.

Histological confirmation of the diagnosis requires a biopsy which can be potentially hazardous in a patient with systemic amyloidosis because there is an

increased risk of bleeding since amyloid deposits are usually present in the walls of small blood vessels throughout the body. In addition, deficiency of clotting factors IX and X which occurs occasionally in AL amyloid must be sought. Biopsy of a major affected organ such as the kidney or heart is most likely to give a positive result but is invasive. It may therefore be preferable to biopsy a more accessible site especially in patients in whom the index of suspicion is low. Commonly used procedures include fine needle aspiration of fat [51] and biopsy of the rectum or labial salivary glands [52] which in experienced hands should provide positive results in at least 80% of patients with systemic amyloidosis [53]. However, a negative result does not exclude the diagnosis and the positive yield in any of the above procedures may be substantially reduced in non-specialist centres.

Histological

Congo red staining

The unique optical property of Congo red stained amyloid has been shown to be dependent on the β-pleated sheet configuration in the native protein [54], allowing planar dye molecules to fit edgewise into the face of the pleated sheets with their long axes in the axis of the filament. Under cross polarised light, the birefringent Congo red crystals show colours that vary from red in thick crystals through orange and yellow to green in thinner crystals. Since the original method of Congo red staining was first described [55], it has undergone several modifications to improve its sensitivity, specificity, and reliability [4].

Other methods of detection of amyloid include fluorescent stains, e.g., thioflavin T or S, and metachromatic stains such as crystal violet. The fluorescent dyes, because of their high sensitivity, may be helpful in screening tissues for amyloid deposits but positive results should be confirmed by more specific methods.

Sensitivity and specificity

The optical properties of Congo red stained amyloid may be affected with loss of green birefringence in very thin or very thick sections. A thickness of 5-10 μm is optimal. Congo red staining of amyloid is more intense in tissues fixed in alcohol or Carnoy's than those fixed in formalin but this may lead to a higher incidence of false positive results. Staining of non-amyloid components may be removed by alcohol and this effect is enhanced by the addition of NaOH. False positive staining may occur with collagen and this may be avoided by saturation of staining solution with NaCl. Congo red solutions are unstable and should be freshly prepared every 2 months. Good microscopic technique, knowledge of the variables involved in Congo red staining and inclusion of positive and negative controls in every staining run is therefore critical to the sensitivity and specificity of the technique.

Classification of amyloid proteins on the basis of Congo red staining

Congo red staining after autoclaving or treating the tissue with potassium permanganate or alkaline guanidine has been used to subclassify amyloidosis [56]. Autoclaving the tissues at 120°C for 30 minutes causes protein AA to lose its affinity for Congo red. Prolongation of autoclaving to 120 minutes abolishes the congophilia of protein AL, but prealbumin-related amyloid show little or no change. Treatment of the tissue with potassium permanganate causes protein AA and β2-M to lose their affinity for Congo red. Protein AA fails to stain with Congo red after treatment with alkaline guanidine for 1 min and protein AL and senile systemic amyloid after 2 hours. However, the general availability of immunohistologic methods which can be used to identify and classify amyloid proteins in tissues with great precision renders obsolete attempts at differentiating amyloid proteins by differential staining through the demonstration of temperature and permanganate sensitivity.

Immunohistologic methods for detection and classification of amyloid

The development of antibodies against amyloid proteins has helped in the identification and classification of amyloid fibrils in tissue sections. Both immunofluorescence (Figure 1.1) and immunoperoxidase techniques are used. The immunofluorescence method requires the use of frozen sections. The immunoperoxidase technique is more sensitive and has the advantage of being able to detect amyloid in formalin fixed paraffin embedded tissues. However, formalin fixation may mask the epitopes of amyloid proteins especially in AL and TTR-related amyloid. Pre-treatment of sections with trypsin may be necessary to break down the cross-linking bridges and "unmask" the epitopes although the process itself may damage and alter the antigenic determinants of the proteins [57]. Antigenicity is much better preserved with frozen sections although tissue architecture is more likely to be disrupted than with fixed sections. In AL amyloid, only about half of the cases are stainable with standard antisera to κ or λ light chains probably because the light chain fragment is usually derived from the variable domain and may contain unique epitopes that are not recognised by the antibodies. Although fibril proteins can be identified immunohistochemically and enable the amyloid to be classified, the demonstration of amyloidogenic proteins in tissues does not on its own establish the presence of amyloid and Congo red staining with green birefringence is always required.

Whilst immunohistochemical staining allows the majority of cases of amyloidosis to be typed, the development of fibril extraction methods means that fibrils can now be extracted from amyloidotic organs or biopsy tissue and fully characterised by protein sequencing and electrospray mass spectrometry.

Under the EM, fibrils are rigid, non-branching and 10-15 nm in diameter. Each fibril is in turn composed of aggregates of 2 to 5 filaments, arranged in a twisted ribbon pattern. However amyloid fibrils cannot always be convincingly identified ultrastructurally, and EM alone is not sufficient to confirm the diagnosis of amyloidosis.

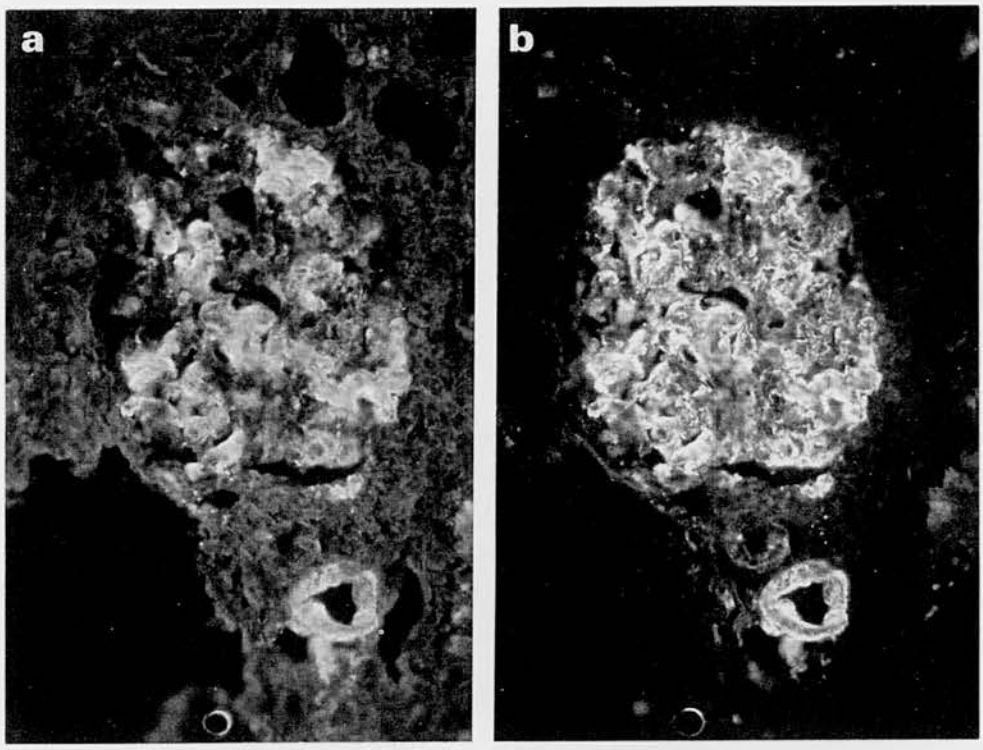

Figure 1.1 Renal glomerular amyloid deposits in a case of AA amyloidosis complicating renal adenocarcinoma. **a.** Immunofluorescence staining with rhodamine-labelled rabbit anti-human AA antibodies. **b.** Same field stained with fluorescein-labelled sheep anti-human SAP antibodies. (x400)

Non-Histological

^{123}I-SAP Scintigraphy

A recent and major development is the use of radiolabelled SAP as a targeting molecule for the *in vivo* diagnosis of amyloid [58-60]. This is based on the principle that SAP is a universal constituent of all amyloid deposits [35] and once injected into the circulation, radiolabelled SAP will localise and bind to amyloid deposits in a specific manner and in proportion to the quantity of amyloid present. SAP is labelled with ^{123}I, a medium energy short half-life pure γ emitter for whole body scintigraphic imaging and with ^{125}I for metabolic turnover studies. ^{123}I-SAP scintigraphy is sensitive and specific

for the *in vivo* diagnosis of amyloid with positive images obtained in over 90% of patients with systemic amyloidosis [61]. In addition, scintigraphy provides information on the tissue distribution of amyloid especially in sites not normally available for biopsy (Figure 1.2) and this is particularly important in determining prognosis and assessing patients for organ transplantation which is relatively contraindicated in patients with a large total body amyloid load. It may be used for the monitoring of amyloidosis, providing unique information on the natural history of amyloidosis and the effects of treatment (Table 1.5). SAP scans have now been performed on over 800 patients without adverse effects but is currently restricted in availability to no more than 20 centres worldwide because of the cost and preparations involved in producing clinical grade pure SAP and the isotope, ^{123}I. However, SAP scintigraphy may become more widely available in the near future with the development of genetically engineered SAP and the use of alternative isotopes such as ^{99}Tc which is cheaper and widely available. The chemistry for labelling proteins with a metal nuclide is challenging but the technique have recently been successfully developed and used in SAP scintigraphy [62].

Attempts have been made to develop other specific tracers for imaging amyloid deposits, the most successful of which are studies involving ^{131}I labelled β2–M [63] as a scanning agent in DRA in which the deposits are derived from β2–M. However, the radioactivity involved is high and the technique is suitable only for anuric patients.

Table 1.5 Applications of SAP scintigraphy

Diagnosis and quantification of systemic and some local forms of amyloidosis
Identifying tissue distribution of amyloid deposits - may indicate type
Screening at-risk individuals for sub-clinical amyloid
Monitoring clinical and sub-clinical disease, *i.e.* natural history
Evaluating effects of therapy

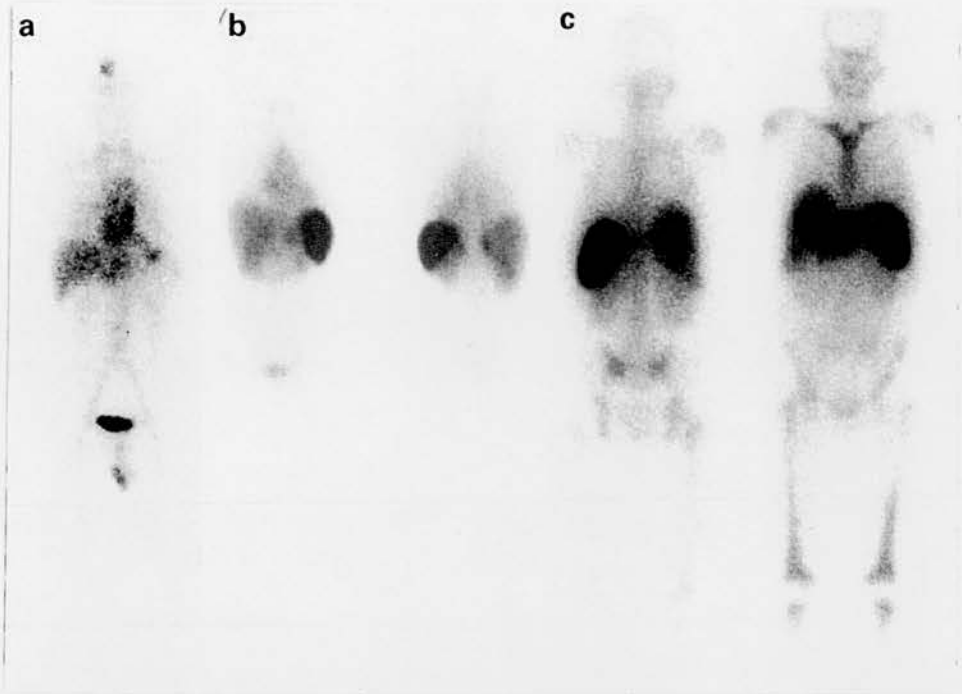

Figure 1.2 Whole body scintigraphs 24 h after intravenous injection of ^{123}I-labelled human SAP. **a,** anterior view of normal control subject showing distribution of residual tracer in the blood pool and radioactive breakdown products in urine in the bladder; note the absence of localisation or retention of tracer anywhere in the body. **b,** anterior (left) and posterior (right) views of patient with juvenile chronic arthritis complicated by AA amyloidosis. There is uptake of tracer in the spleen, kidneys and adrenal glands, a typical distribution of AA amyloid in which the spleen is involved in 100% of cases, kidneys in 75% and adrenals in 40%. **c,** posterior (left) and anterior (right) views of patient with monoclonal gammopathy complicated by extensive AL amyloidosis. There is uptake and retention of tracer in the liver, spleen, kidneys, bone marrow and soft tissues around the shoulder. Note the complete absence of blood pool or bladder signal compared to **a.** This pattern of amyloid distribution revealed by scintigraphy is pathognomonic for AL amyloidosis, bone marrow uptake never having been seen in any other type.

Echocardiography, radiology and gene studies

Echocardiography is the most sensitive non-invasive means of diagnosing cardiac amyloid [64]. Small, concentrically hypertrophied ventricles with generally impaired contraction, dilated atria, homogeneously thickened valves, and 'sparkling' echodensity of ventricular walls are characteristic echocardiographic findings of cardiac

amyloid whilst ECG classically shows low voltage complexes.

Bony involvement by amyloid deposits occurs occasionally in AL amyloid but characteristically and invariably in β2–M amyloid which typically produces multiple periarticular cystic bone lesions on X-ray examination which grow in size and number with increasing duration of dialysis. Commonly affected sites include the carpal bones, femoral and humoral heads, acetabulum, tibial plateau and distal radius, and may result in pathological fractures especially in weight bearing bones [65]. Extraosseous radiologic findings which include swelling of articular and periarticular soft tissue mainly involving joint capsules, synovia and tendons, can be visualised and quantified by ultrasonography [66].

In some patients, AL amyloid is the only feature of an underlying plasma cell dyscrasia with no demonstrable morphological evidence of the clonal population on bone marrow aspiration and trephine biopsy nor of its products such as a paraprotein band or Bence Jones proteins. These tiny populations of plasma cells can now be identified with immunoglobulin gene rearrangement studies by Southern blotting [67, 68] but only if they represent at least 1% of the total cells [67]. Hence, whilst serum or urine protein electrophoresis is used to detect products of the plasma cell clone, immunoglobulin gene rearrangement studies are now available to detect the clone itself.

In hereditary systemic amyloidosis and familial AD, DNA sequencing has identified the gene mutations responsible for the disease and is also used together with other methods such as allele specific oligonucleotide hybridisation to screen relatives who may be carriers of the mutation and therefore at risk of developing the disease.

Clinical Features

Amyloid deposits exert their pathological effects largely through their physical presence, which distorts tissue architecture and hence disrupts normal function. Continued accumulation ultimately leads to organ failure, or the consequences of a space occupying mass lesion. In addition there may be secondary effects due to the presence of amyloid, such as renal interstitial fibrosis.

The spectrum of clinical features resulting from systemic amyloidosis is therefore enormous (Table 1.6). Clinical presentation, distribution and the amount of amyloid varies greatly in different forms of amyloid and even among patients with the same form. However, the features of any particular organ involvement generally follow the same pattern regardless of the fibril type.

Table 1.6 Clinical features of systemic amyloidosis

CLINICAL FEATURES	COMMENTS
Renal disease Proteinuria Nephrotic syndrome Renal impairment Hypertension Haematuria Renal vein thrombosis Nephrogenic diabetes insipidous Renal tubular acidosis	Usually involve glomeruli or less commonly interstitium. Most frequent presenting feature of AA and AL amyloid is proteinuria especially when glomeruli are involved and may result in nephrotic syndrome with risk of renal vein thrombosis. Significantly increased risk of renal failure if proteinuria >2g/day. Hypertension or less commonly haematuria may occur. Renal failure major determinant of prognosis and most common cause of death in AA amyloidosis. Prognosis considerably improved with RRT
Cardiac disease Restrictive cardiomyopathy Biventricular 'diastolic' failure Rhythm disturbances Heart block Orthostatic hypotension	Commonly involved and most important cause of death in AL amyloid. Clinically significant cardiac amyloid in AA more common than previously thought especially in patients maintained on dialysis. May be predominant or sole feature of hereditary systemic amyloidosis. Typically results in restrictive cardiomyopathy which may be subclinical. Conduction disturbance common, causing sudden death. Increased sensitivity to digoxin because of binding to amyloid fibrils.
Respiratory tract disease Dyspnoea Haemoptysis Stridor Cough	Usually seen in AL amyloid. Often asymptomatic despite diffuse or nodular involvement throughout lungs. May rarely cause pulmonary hypertension
Gastrointestinal disease Macroglossia (AL) Dysphagia Diarrhoea, constipation Malabsorption Intestinal pseudo-obstruction. perforation Hepatomegaly, splenomegaly	Seen in all types of systemic amyloidosis. Diffuse infiltrative pathology with mass lesions may result in malabsorption, pseudo-obstruction and even perforation. More commonly results in anorexia, nausea, vomiting and diarrhoea. Cholestasis and jaundice are features of hepatic amyloid which may rarely cause portal hypertension or liver failure, whilst splenic amyloid can cause functional hyposplenism. Rarely, spontaneous rupture of liver or spleen may occur.

Skin disease Bruising, purpura Plaques, papules, nodules Alopecia, nail changes	Most commonly seen in AL amyloid
Nervous system disease Peripheral neuropathy Autonomic neuropathy Carpal tunnel syndrome Enlarges peripheral nerves Myopathy	Mixed sensorimotor peripheral neuropathy occurs occasionally in AL, commonly in DRA (CTS most common presenting symptom), and classically in FAP. Neuropathy especially in FAP is relentlessly progressive and frequently involve the autonomic nervous system as may occur in AL
Joint disease Arthropathy Effusions Destructive spondyloarthropathy	Occurs occasionally in AL but invariably in DRA, characterised by arthralgias of large and medium sized joints (shoulders most frequently affected) and destructive spondyloarthropathy especially in the cervical spine.
Miscellaneous Lymphadenopathy Isolated Factor IX and X deficiency Goitre Sicca syndrome Vitreous opacities Unexplained acute phase reaction (APR)	Rare clotting factor deficiencies seen only in AL which may also occasionally present with recurrent lymphadenopathy. Goitre due to amyloid infiltration occurs in most types of systemic amyloidosis. Sicca syndrome and vitreous opacities characteristically seen in FAP. Unexplained APR in association with systemic amyloid usually due to occult malignancy or Castleman's disease.

RRT, renal replacement therapy; DRA, dialysis-related amyloid; CTS, carpal tunnel syndrome; FAP, familial amyloid polyneuropathy.

Chapter 2 - Materials and Methods

General Reagents

Buffers

EDTA	Equimolar mixture pH 7.5 of disodium salt, pH 4.0 of tetrasodium salt, pH 11.0 of ethylene diamine tetra-acetate.
PBS	Phosphate buffered saline, pH 7.3 (Oxoid Ltd, Basingstoke, Hants)
PBS-serum-EDTA	Sterile isotonic PBS, pH 7.4, containing Na_3EDTA 2 mg/ml (Limclair, Sinclair Pharmaceuticals Limited, Godalming, Surrey) and 5% v/v heat treated (560C, 30 min) accredited serum (National Blood Transfusion Service, Edgware)
TBS	Tris buffered saline, pH 7.6, 50 mM Tris, 0.85% w/v sodium chloride, pH adjusted with HCl
TC	0.01 M Tris, 0.138 M NaCl, 0.002 M $CaCl_2$, 0.1% w/v
NaN_3,	pH 8.0
TCB	TC containing 1% w/v BSA (Sigma)
TE	0.01 M Tris, 0.138 M NaCl, 0.01 M EDTA, 0.1% w/v NaN_3, pH 8.0

Proteins

Pure human SAP for clinical scintigrahic studies was provided by Professor M B Pepys, having been isolated to more than 99% purity by affinity chromatography as described [69]. HSA and BSA were obtained from Sigma.

Antisera

Rabbit antisera against human SAP and SAA were raised by immunisation with pure isolated proteins emulsified in Freund's complete adjuvant (CFA; Difco Labs, Surrey) followed by booster injections in incomplete Freund's adjuvant (ICFA, Difco) and provided by Professor M B Pepys. The following polyclonal antisera raised in rabbits or sheep against known human amyloid proteins were commercially available:

anti-κ light chains
anti-λ light chains
anti-IgG
anti-β2–M
anti-TTR
anti-apoAI
anti-fibrinogen
anti-lysozyme

Sample preparation for histological analysis

Frozen sections

Fresh material for histology were snap frozen with dicholorodifluoromethane (Arcton 12 I.C.I., Cheshire, U.K.) and then mounted on cork disks in O.C.T. (Tissue-Tek, Raymond Lamb, London, U.K.) embedding medium. Frozen blocks were stored at -80°C. Frozen sections of 6 μm thickness were cut on a cryostat

microtome (-20°C) and air dried for 60 min prior to staining or stored at -80°C until required.

Paraffin sections

Tissue was fixed for 24 hours in 10% buffered formalin, embedded in paraffin and 6 μm sections were kindly cut on to PLL coated slides by the Department of Pathology, Hammersmith Hospital, for staining and immunohistochemical studies.

Amyloid fibril preparations

A 20 μl drop of the solution was placed on a poly-L-lysine coated microscope slide, air dried, then fixed with 95% ethanol v/v for 3 min.

Histological methods

Congo red staining

For histological frozen sections Congo red staining was done by the method of Puchtler et al [4]. The slides were rehydrated with TBS for 5 min and counterstained with Coles Haematoxylin for 10 min which was then "blued" with tap water. Freshly filtered Congo red (Raymond Lamb) prepared in alcoholic saturated salt was made alkaline with 1% w/v sodium hydroxide and the slides immersed in this for 30 min. After 3 rinses with 100% ethanol they were placed in Histoclear® (National Diagnostics, New Jersey, U.S.A.) for 5 min and then mounted under Histomount® (Natural Diagnostics). Following drying for at least 1 hr the mounts were examined under a Leitz dialux 20 EB microscope (Wetzlar, Germany). Paraffin sections were dewaxed with Histoclear® and ethanol and rehydrated with water before Congo red staining was performed. Fibril preparations were stained only with Congo red but were otherwise processed similarly, with positive control material always processed in parallel.

Typing of amyloid by immunohistochemistry

Peroxidase-anti-peroxidase method

Immunohistochemical staining by the PAP method was performed on paraffin fixed sections which was first deparaffinised and rehydrated in the usual manner [57]. Sections were then incubated overnight at 4°C with the 1° anti-serum at established dilution (usually 1:500 to 1:10 000) in 1% TBS containing normal serum from host of 2° antibody. Slides were then washed on a rotating platform, twice with TBS containing Triton X-100 (BDH Chemicals Limited, Poole, U.K.) 0.005% (v/v) (TBS-T) for 5 min followed by TBS alone for a further 5 min. Sections were then incubated for 60 min at room temperature with 2° antibody at established dilution (usually 1:50) in TBS with 5% normal serum from tissue host. After washing as above to remove unbound antibody, sections were incubated for 30 min at room temperature with peroxidase anti-peroxidase antibody complexes at established dilution (usually 1:100) in TBS with 1% normal serum from host of 2° antibody. After another wash cycle, bound enzyme was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma 5637) (Sigma Chemical Company Limited, St. Louis, Missouri, U.S.A.) 0.05% w/v in TBS containing 10 mM imidazole (BDH), pH 7.4 to 7.6 and 0.02% v/v H_2O_2 (Taab Laboratory Equipment Limited, Reading, U.K.) as substrate. Sections were immersed for 5 to 10 min, rinsed with dH_2O, then counterstained lightly with Coles Haematoxylin for 30 seconds. After blueing in running tap water and rinsing with dH_2O, sections were dehydrated and mounted in the usual manner.

Indirect immunofluorescence

Typing of amyloid protein by IIF was performed only on frozen sections which were first rehydrated with TBS and the background blocked for 15 min at room temperature with 10% normal serum from the species donating the 2° antibody. Sections were then incubated for 30 to 60 min at room temperature with 1° antibody at established dilution in buffer containing 1% normal serum from host of 2° antibody. After washing as above to remove unbound antibody and to reduce non-specific binding due to ionic or electrostatic forces, sections were incubated for

30 min at room temperature with 2° antibody (anti-Ig of the species providing the 1° antibody - conjugated with FITC or RITC) at established dilution (usually 1:50) in buffer containing 5% normal serum from the tissue host to negate any reaction between 2° antibody and tissue Ig. The sections were then washed and mounted in Citifluor (Citifluor Limited, London, U.K.) under coverslips sealed with varnish, and examined immediately with a Leitz Orthoplan microscope with water immersion lenses. Photography was performed with a Leitz Orthomat system on Kodak ASA400 slide film.

Antisera and controls

Antibodies directed against a range of known amyloid proteins which were all commercially available were used. The following controls were also included:

- Control tissue known to be positive.
- Replacing first antiserum with 1% of normal serum of the species of the second antibody.
- Replacing first antiserum with normal rabbit serum diluted as much as the antiserum.
- Replacing the first antibody with antibody absorbed with specific antigen.

Interpretation of results

Tissue structures stained with Congo red in an alcoholic alkaline salt solution were regarded as showing positive staining if they showed apple green birefringence when viewed under cross polarised light (Figure 4.1, Figure 4.3). SAP is normally present in the GBM and as a mantle on the elastic fibres throughout the body, SAP in other sites is abnormal. To be of diagnostic significance the fluorescence of the other stains, e.g. anti-SAA should be titratable: positive in the known control tissue, and negative in the replacement controls.

The type of amyloid fibril is readily determined by immunohistochemical methods. The widely used potassium permanganate method [56, 70] in which some

types of amyloid, notably AA, lose their capacity to bind Congo red when pre-treated with this agent is not specific [14]. Antisera to SAP/AP, AA, β2-M, transthyretin, apoAI, lysozyme, fibrinogen, kappa and lambda light chains are all commercially available and may be used to stain fixed or snap frozen tissue. Variable and poor results are obtained in AL amyloid [71, 72] as the light chains are derived predominantly from the variable (V_L) domain and hence differ from case to case. Anti-transthyretin rarely stains the deposits of senile systemic amyloid though good results may be achieved with hereditary transthyretin amyloid.

Isolation of amyloid fibrils from gram quantities of tissue

Organs from patients with amyloidosis obtained at post-mortem or during an operation were stored at -20°C and used for fibril extraction by the method of Pras et al [8]. Approximately 30 gms of tissue were homogenised in 300 ml of 0.14 M NaCl and 0.01 M EDTA with 0.1% w/v NaN_3 using an Ultraturrax homogeniser (Sartorius Instruments Ltd, Surrey, UK) and then centrifuged at 15 000 g for 30 min. The process was repeated 8 to 15 times, discarding the supernatant each time, until the A_{280} of the supernatant stabilised at < 0.05. The pellet was then homogenised with pure distilled water, centrifuged at 20 000 g for 30 min and the process repeated a further 3 to 4 times.

A_{280}, corrected by subtraction of A_{320} (for light scattering), was measured on each salt and water supernatant. Material from the DW washes with the greatest A_{280}, (usually the second wash with A_{280} of 1.5 to 3) was dried on a microscope slide and stained with Congo red to confirm the presence of amyloid fibrils. Fibrils extracted by this method were either lyophilised using an Edwards E F4 Modulyo freeze dryer (Edwards High Vacuum, Crawley, Sussex, UK) or kept in DW at 4°C, where they remained in solution for periods of at least 3 months.

Isolation of amyloid fibrils from milligram quantities of tissue

The standard water extraction method [8] for isolation of amyloid fibrils from tissue involves numerous preliminary homogenisations in saline, during which, in our experience, significant losses of fibrils may occur. In order to maximise recovery from tiny milligram quantities (1 to 2 mg) of tissue obtained at biopsy, the tissue was initially homogenised in 0.5 ml of distilled water to extract the fibrils immediately [10]. After centrifugation at 15,000 *g* for 10 min and separation of the supernatant, the process was repeated twice more and the supernatants were pooled together. The amyloid fibrils were then selectively precipitated by adding sodium chloride to a final concentration of 0.2 mol/l, leaving essentially all other components of the water extract in solution in the saline. The suspension was centrifuged at 15,000 *g* for 10 min and the presence of amyloid fibrils was confirmed by drying 10 µl of resuspended material on a poly-L-lysine coated slide, fixing with 10% formalin/90% ethanol and staining with Congo red [4]. The protein composition of the fibril pellet was analysed by SDS 8-18% gradient PAGE.

Polyacrylamide gel electrophoresis

Gradient PAGE analysis of reduced SDS treated proteins was performed in 8-18% gradient gels (Pharmacia ExcelGel™) run in accordance with manufacturer's instructions. Standard globular proteins used as high molecular weight markers were myosin 205 kDa and β-galactosidase 116.5 kDa. Low molecular weight marker polypeptides were phosphorylase B 106 kDa, bovine serum albumin 80 kDa, ovalbumin 49.5 kDa, carbonic anhydrase 32.5 kDa, soybean trypsin inhibitor 27.5 kDa and lysozyme 18.5 kDa (Bio-Rad Laboratories, Richmond, U.S.A.). Gels were stained with Coomassie Blue according to the manufacturer's instructions.

Immunoblotting

Immunodetection of fibril subunit proteins was performed by Western blotting. Isolated amyloid fibrils or fragments of delipidated amyloid laden subcutaneous tissue were solubilised by boiling in 10 mM Tris, pH 8.0 containing 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 2.5% w/v SDS and 1 mM EDTA. Amyloid fibril subunit peptides were separated by SDS 8-18% gradient PAGE (Excelgel™, Pharmacia) and transferred to pure 0.2 μm nitrocellulose membrane (Scleicher and Schull A/G, Anderman and Co. Ltd., Kingston-upon-Thames, Surrey, UK) by electroblotting at 0.8 mA/cm^2 for 30 minutes using the Pharmacia Novablot™ semidry blotting apparatus with continuous buffer system according to the manufacturer's instructions. Immunostaining was performed with rabbit anti-human immunoglobulin (Dako), horseradish peroxidase-labelled affinity purified goat anti-rabbit IgG (Dako) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Company, St Louis, MO, USA) according to the Bio-Rad immunoblot protocol (Bio-Rad Labs Ltd., Hemel-Hempstead, Herts, UK).

Isolation of DNA and Amplification and Sequencing of TTR Gene

DNA was extracted from whole blood [73] and TTR exons were amplified by the polymerase chain reaction (PCR) using *taq* polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT) with the following cycling conditions: 1 cycle of 94°C, 5 minutes; 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and a final step of 72°C for 10 minutes. For exon 1 intron sequences, (5′ to 3′), CAGCAGGTTTGCAGTCAGAT and GGTACCCTTGCCCTAGTAAT were used, for exon 2, CAATTTTGTTAACTTCTCACG and CAGATGATGTGAGCCTCTCTC; exon 3, CCTCCATGCGTAACTTAATCC and TAGGACATTTCTGTGGTACAC; exon 4, TGGTGGAAATGGATCTGTCTG and TGGAAGGGACAATAAGGGAAT.

PCR products (100 μL) were purified by size fractionation on a Nusieve agarose gel (FMC, Rockland, ME). The band was extracted using Magiprep

columns (Promega, Madison, WI), recovered by ethanol precipitation and dissolved in 12 μL of distilled water. Six μL was then used in the sequencing reaction for each primer.

The sequencing reaction was modified from Casanova et al [74]. A reaction mix containing 2 μL of sequencing buffer, 2 μL of primer (100 ng/μL) and 6 μL of template was boiled for 2 minutes, before freezing in a dry ice/methanol bath for 15 seconds, and then adding 5 μL of Mastermix. Just after the mixture thawed, 3 μ L was added to 2.5 μL of each of the four dideoxynucleotides and the termination reaction was then incubated at 37°C for 2 minutes before addition of 4 μL of stop solution. The same primers were used for PCR and sequencing, except that for exon 4 the primer (5′-3′), CTCGTCCTTCAGGTCCACTG, was used since, for unknown reasons, poor sequence was obtained with the exon 4 PCR downstream primer.

Isolation and radiolabelling of SAP

SAP, isolated from the serum of a single accredited blood donor provided by the National Blood Transfusion Service (Edgware, London) was purified to greater than 99% purity under strictly sterile conditions and was stored sterile and frozen at -70°C. For each study 1 mg of pure SAP was oxidatively labelled with 1000 Mbq of $Na^{123}I$ (Amersham International), N-bromosuccinimide being used to achieve 85-95% incorporation. Free ^{123}I was removed by gel filtration on Sephadex G25 column (PD10, Pharmacia, Milton Keynes, U.K.) which was equilibrated with 5% v/v heat treated (56°C, 30 min) accredited serum in phosphate buffered saline (PBS), pH 7.4, containing Na_3EDTA 2 mg/ml (PBS serum-EDTA). The column was then promptly eluted with 5 ml PBS serum-EDTA which served to quench the reaction and to separate iodinated protein from free radioiodine. After this gel filtration step more than 95% of the radioactivity was precipitable by 10% w/v tricholoroacetic acid. The labelled protein was then passed through a sterile 0.22 μm filter into a sterile vial to provide sufficient material for 5 patient studies.

Imaging

Each subject received by intravenous injection approximately 200 μg of SAP bearing approximately 190 Mbq of ^{123}I, corresponding to an effective dose equivalent of 3.8 mSv and comparable with that from an intravenous pyelogram or barium meal study. Thyroid uptake was blocked by administration of potassium iodide 60 mg twice daily for 2 days before the study and for the subsequent 5 days. Anterior and posterior whole body and abdominal imaging was performed in each individual 24 hours after injection of ^{123}I-SAP by means of an IGE-Starcam γ-camera (IGE Medical Systems, Slough, U.K.). In dialysis patients, static images of the wrists, shoulders and knees were also taken.

Metabolic studies

Venous blood samples were taken at intervals (10, 20, 30 min, 6 and 24 hrs) after ^{123}I-SAP injection. Radioactivity precipitable by trichloroacetic acid was determined in each serum sample as a proportion of the injected dose.

Chapter 3 - Hereditary Renal Amyloidosis

Hereditary renal amyloidosis due to a new apolipoprotein AI Trp50Arg variant in an Australian family

Introduction

Hereditary non-neuropathic systemic amyloidosis, first described in a kindred reported by Ostertag [75, 76] is a rare autosomal dominant condition in which amyloid deposition in the viscera, especially the kidneys, leads inexorably to organ failure. It can present at any age from the second to the sixth decade and there is considerable variation both within and between families in the presentation, pattern of organ involvement and rate of progression. Single residue substitution variants of three different proteins, apolipoprotein AI (apoAI) [19, 77], lysozyme [21] and fibrinogen α-chain [22, 78], encoded by point mutations in the respective genes, have been identified as the causes of this syndrome in different families. However there is little relationship between the amyloid fibril protein or its particular variant and the clinical manifestations. Whilst inheritance of an amyloidogenic mutation is necessary for development of the disease in each case, other factors evidently play an important role in determining the distribution and clinical effects of the amyloid deposits.

A previously undescribed kindred with hereditary non-neuropathic systemic amyloidosis in which the amyloid fibrils from the proband consisted of *N*-terminal fragments of a variant of mature apoAI with arginine in place of tryptophan at position 50 is described here. The two previously reported amyloidogenic variants of apoAI also contained arginine substitutions for uncharged residues, Gly26Arg, which also causes neuropathic amyloid in one family [18, 42], and Leu60Arg [19] respectively. There is no such pattern of residue substitutions among the known amyloidogenic variants of other proteins [1, 14]. There may thus be a common mechanism of apoAI amyloidogenesis resulting from acquisition of the extra arginine residue *per se* and/or the additional positive charge it confers.

Methods

Kindred

The proband was an only child born in 1947 to Ashkenazi Jewish parents. His mother is alive and well in her seventies. His father died in 1968, aged 45, with massive hepatic and renal amyloidosis confirmed histologically. The father's parents and all his siblings were murdered in the Nazi holocaust and no further previous family history of amyloid is available. The proband's children are well and have not been investigated.

The proband presented in 1981, aged 34, with microscopic haematuria detected on routine urinalysis. He also had slight hepatomegaly and raised serum alkaline phosphatase, 272 U/l (upper limit of normal 260). There was trace proteinuria and his renal function was moderately impaired, creatinine 129 mmol/l (normal 30-120). At routine health screening in 1973 his urinalysis and serum biochemistry had been normal. The microscopic haematuria persisted, renal function and liver function tests slowly deteriorated, and he developed intermittent explosive diarrhoea. Renal and small and large bowel biopsies in 1983 all contained extensive amyloidosis. In 1989 *in vivo* scintigraphy with ^{123}I-labelled serum amyloid P component [59, 79] demonstrated massive hepatic and splenic amyloidosis (Figure 3.1). Although he continued to work full-time, renal and hepatic function deteriorated slowly but progressively until 1991 when he commenced peritoneal dialysis. Proteinuria had increased to 2.47 g/day at that time but he was never hypoalbuminaemic or nephrotic. Hepatomegaly had increased, splenomegaly had appeared and he suffered chronic diarrhoea and malabsorption. However, there was no clinical evidence of peripheral or autonomic neuropathy and nerve conduction was normal until late in the course, when the changes were compatible with advanced renal failure. There was also no clinical cardiac involvement and echocardiography was normal. Two months after successful renal transplantation in March 1992, he died with an overwhelming cytomegalovirus infection and liver failure.

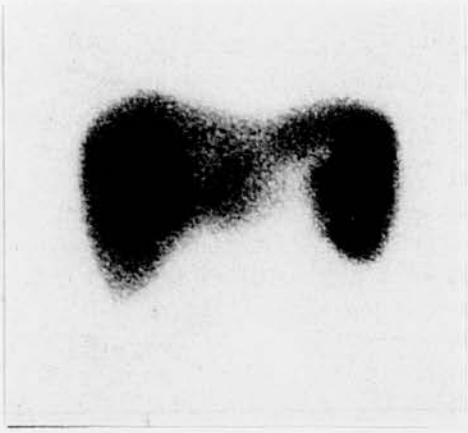

Figure 3.1 Anterior scintigram of abdomen in the proband 24 h after intravenous injection of ^{123}I-SAP. There is massive uptake in the liver and spleen and no blood pool background, indicating a heavy whole body amyloid load.

Identification of the amyloid fibril protein

Immunohistochemical staining, SDS-PAGE analysis, isoelectric focusing and immunoblotting were all performed exactly as described in our report of the apoAI Leu60Arg variant [19]. The only amyloidotic tissue available was a needle biopsy of liver and amyloid fibrils were therefore isolated from 50 mg of tissue, using the modified water extraction procedure recently reported [10].

Identification of the mutation in the apoAI gene

DNA was extracted from the proband's muscle tissue [80] and three fragments comprising the coding region of the apoAI gene were amplified by PCR and subjected to nucleotide sequencing [19], or cloned into pGEM.T (Promega, Madison, WI, USA) and sequenced [81]. The PCR product of exon 4 was also digested with *MspI* and the fragments analysed by polyacrylamide gel electrophoresis [82].

Characterisation of the amyloid fibril subunit protein

Isolated amyloid fibrils were dissolved in 6 M guanidine hydrochloride/0.5 Tris, pH 8.5 and fractionated by FPLC gel filtration on Superose 12HR (Pharmacia) in 4 M guanidine hydrochloride/0.05 Tris, pH 8.5. The single major eluted peak was dialysed against water and lyophilised before analysis by electrospray mass spectrometry [19]. For polypeptide analysis the whole amyloid fibril isolate was run in SDS-PAGE, transferred to a Problott membrane (Applied Biosystems) and the single main band at ~10 kDa was sequenced using fast cycle chemistry in a modified Applied Biosystems 477A system [83]. The band was also excised from the gel, digested with lysyl endopeptidase and the products analysed by matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry [19]. The digest was then fractionated on HPLC, the main peaks were characterised by MALDI-TOF, and the variant peptide was subjected to automated sequencing [83].

Results

Identification of the amyloid fibril protein as apoAI

The amyloid deposits in a liver biopsy taken during the proband's final illness stained immunospecifically with antibodies to apoAI. Amyloid fibrils isolated from the liver tissue gave a single major protein band, running at about 10 kDa in SDS-PAGE (Figure 3.2), that stained specifically on immunoblotting with antibodies to apoAI. The first 28 amino acid residues of this band were determined by automated Edman degradation after blotting from SDS-PAGE and comprised the *N*-terminal sequence of mature wild type apoAI. However immunoblotting after isoelectric focusing of the proband's delipidated plasma proteins demonstrated a variant apoAI species with a single extra positive charge, in addition to wild type apoAI (Figure 3.3).

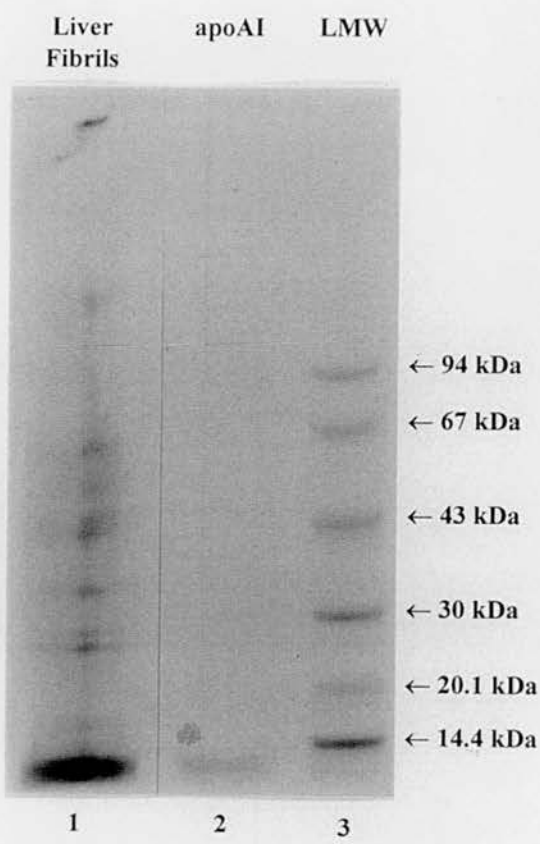

Figure 3.2 SDS 8-18% PAGE analysis of amyloid fibrils isolated from the proband's liver (lane 1). The major band at about 10 kDa migrates to the same position as the purified subunit of the amyloid fibrils from the spleen of a patient with Leu60Arg variant apoAI amyloidosis [19] (lane 2). Molecular weight markers were run in lane 3.

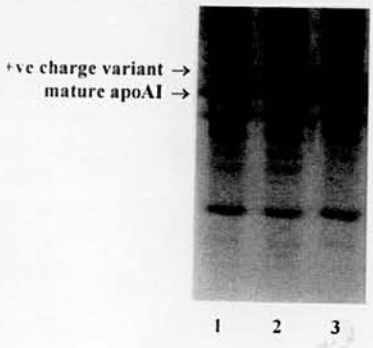

Figure 3.3 Isoelectric focusing and immunoblotting of plasma apoAI showing the variants with one extra positive charge. Lane 1, proband; lane 2, known case of Leu60Arg apoAI amyloidosis [19]; lane 3, normal control.

Identification of the apoAI gene mutation

The exons of the proband's apoAI gene were amplified by PCR, cloned and sequenced, demonstrating that he was a heterozygote for a T→C transition changing the codon for residue 50 in the mature protein from TGG (Trp) to CGG (Arg) (Figure 3.4), compatible with the observed charge variant in the plasma. The mutation creates a new restriction site and this was confirmed by analysis of the fragments after digestion of the PCR product with *MspI* (Figure 3.4).

Characterisation of the amyloid fibril subunit protein

Electrospray mass spectrometric analysis of the purified amyloid fibril subunit protein, apparent mass about 10 kDa, demonstrated three species (Figure 3.5) corresponding to the *N*-terminal 86, 92 and 93 residues of mature apoAI variant with a single Arg for Trp substitution (Table 3.1). In each case the measured mass exceeded the predicted value by ~16 Da, compatible with oxidation of Met86 to methionine sulphoxide as we have previously observed in fibrils formed from the apoAI Leu60Arg variant [19]. There was no evidence of material corresponding to the wild type apoAI sequence.

After incubation of the fibril subunit protein with lysyl endopeptidase the whole digest was analysed by MALDI-TOF mass spectrometry and all peptides resolved from the matrix with mass greater than about 1 kDa were detected. Their masses corresponded to those predicted from the variant apoAI sequence (Table 3.2). In particular the peptide of measured mass 1582 Da, corresponding to the fragment of predicted mass 1582.7 Da containing the Arg for Trp substitution at residue 50, was clearly seen, whilst there was no sign of a peptide of mass 1612.8 Da expected from the wild type sequence. The peptide of expected mass 1307.4 Da was detected at 1325 Da, the increment of 17.6 Da being compatible with the oxygen atom involved in sulphoxidation of Met86, given the lower precision of TOF compared to electrospray mass spectrometry.

Finally the lysyl endopeptidase digest was fractionated by HPLC and the fractions were screened by uncalibrated MALDI-TOF mass analysis to identify the

variant peptide for Edman sequencing, and only Arg was detected at the position corresponding to residue 50 in mature apoAI.

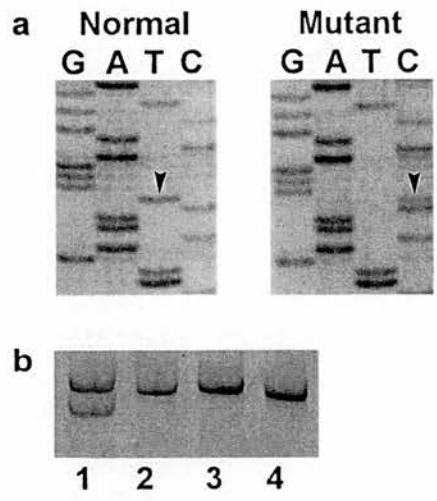

Figure 3.4 ***a,*** Nucleotide sequence of plasmid clones of part of exon 4 of the apoAI gene of the proband. Partial sequence of the normal and variant alleles demonstrates the T→C transition in codon 50 that encodes Arg in place of Trp. ***b,*** Restriction fragment analysis. The mutation introduces an *MspI* restriction site and digestion of the PCR products of exon 4 in the proband (lane 1) yielded an uncleaved 391 base pair band, corresponding to the normal allele, and cleaved products of 339 base pairs (lane 1) and 52 base pairs (not seen). Digestion of a normal control (lane 3) gave a single band running in the same position as the undigested material from either the patient (lane 2) or the control (lane 4).

Table 3.1 Amyloid fibril subunit analysis by electrospray mass spectrometry

Peak	Measured mass (Da)	Predicted apoAI Trp50Arg fragment	*Calculated mass (Da)
A	9,903.1	Residues 1-86	9,905.0
B	10,606.6	Residues 1-92	10,606.7
C	10,706.2	Residues 1-93	10,705.9

*Including 16 Da for the extra oxygen atom incorporated by sulphoxidation of residue Met86

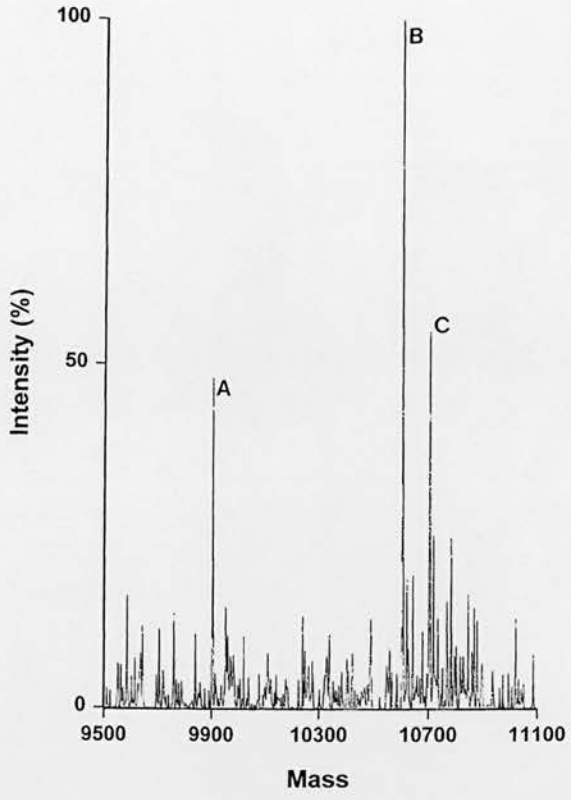

Figure 3.5 Electrospray mass spectrum of the purified 10 kDa amyloid fibril subunit protein. The precise masses and assignments of the three species, A, B and C are shown in Table 3.1.

Variation in clinical expression

Table 3.3 summarises information on all currently known kindreds with hereditary apoAI amyloidosis, including two families under our care who have not previously been reported, as well as the present case. Although all patients have had extensive visceral deposits, there are striking variations in presentation, manifestations and course between different kindreds, even those with the same causative mutation and even between members of the same family (Table 3.3). For example the initial clinical presentation ranges from the second to the sixth decade, and only the original Iowa family suffered from peripheral neuropathy. Renal failure is almost universal though the rate of progression varies greatly, as does the occurrence and severity of cardiac involvement. On the other hand some individuals from families with different mutations have followed a very similar course.

Table 3.2 Polypeptide analysis of the amyloid fibril subunit

Peptide	Residues	Predicted sequence	Predicted mass (Da)	Whole digest mass (Da)	HPLC separated peptides Mass*(Da)	HPLC separated peptides Automated sequence
1	1-12	DEPPQSPWDRVK	1453.6	1454	1456	nd
2	13-23	DLATVYVDVLK	1235.4	1238	1264	nd
3	24-40	DSGRDYVSQFEGSALGK	1815.9	1815	1816	nd
4	41-45	QLNLK	614.7	ND	nd	nd
5	46-59	LLDN**R**DSVTSTFSK	1582.7	1582	1580	LLDN**R**DSVTSTFSK
6	60-77	LREQLGPVTQEFWDNLEK	2202.4	2205	2205	nd
7	78-88	ETEGLRQEMSK	1307.4	1325	nd	nd
8	89-94	DLEEVK	731.8	ND	nd	nd

The main subunit band running at about 10 kDa in SDS-PAGE of isolated amyloid fibrils was digested with lysl endopeptidase and the whole digest analysed by MALDI-TOF. The digest was then fractionated by HPLC and uncalibrated mass analysis (*) was used to identify the variant peptide for sequencing. The predicted sequence is that of mature apoAI Trp50Arg, as indicated by the gene sequence; the variant residue is shown by the boldface **R** in peptide 5.
ND, not detected; nd, not done.

Table 3.3 Clinical features of hereditary apoAI amyloidosis

Kindred (ref) {no of cases}	Ethnic origin	ApoAI variant	Clinical presentation {age range}	Main clinical features	Outcome
Iowa[84] {14}	British	Gly26Arg	Peripheral neuropathy {26-44}	Neuropathy, peptic ulcer, renal failure, extensive visceral amyloid	Death after 1-12 years
Boston[77] {2}	Scandinavian	Gly26Arg	Hypertension {25-?}	Renal failure, extensive visceral amyloid, no neuropathy	Death after 18 years
Canada[85] {4}	British	Gly26Arg	Hypertension, haematuria {26-46}	Slowly progressive renal failure, asymptomatic visceral amyloid	End stage renal failure after up to 20 years
Irish* {2}	British	Gly26Arg	Hypertension {38-43}	Progressive renal failure, asymptomatic visceral amyloid	Death or end stage renal failure after 10 years
English A[19] {5}	British	Leu60Arg	Hypertension, renal failure or organomegaly {25-45}	Progressive renal failure, massive asymptomatic visceral amyloid	All alive up to 12 years from presentation; one case 7 years after renal transplantation
English B* {2}	British	Leu60Arg	Renal and cardiac failure {25-60}	Progressive renal and cardiac failure in daughter, asymptomatic visceral amyloid in father	Heart and renal transplant in daughter; death from stroke at age 64 in father
Jewish {2}	Ashkenazi	Trp50Arg	Haematuria {34-35}	Progressive renal failure, massive visceral amyloid	End stage renal failure, death after 10 years

*Unpublished

Discussion

The proband's father died of systemic amyloidosis for which no primary cause was identified. The proband similarly had no evidence of any other disease known to be complicated by amyloidosis, strongly suggesting that he suffered from hereditary amyloidosis. Furthermore the proband's amyloid fibrils were composed exclusively of a variant apoAI protein, Trp50Arg, encoded by a mutant allele of the apoAI gene for which he was a heterozygote. This variant has not been found previously, even in very extensive population studies [86]. Analogous findings in patients with the Gly26Arg [18, 42] and Leu60Arg apoAI mutations [19], and concordance in these more extensive kindreds with development of amyloidosis, identify the mutant genes as the cause of the disease.

The mechanism by which single residue substitutions cause a normally soluble protein to be deposited in an insoluble, abnormal, form as amyloid fibrils that cause devastating disease *in vivo* is obscure. However it is notable that the three known amyloidogenic variants of apoAI all contain the cationic amino acid arginine in place of non-charged residues. In contrast the pathogenic variants of other amyloidogenic proteins involved in hereditary or acquired amyloidosis, including transthyretin, cystatin C, gelsolin, β-amyloid precursor protein, prion protein, lysozyme, fibrinogen α-chain, serum amyloid A protein, and immunoglobulin light or heavy chains, show no discernible pattern of residue substitutions [1, 14]. No three dimensional structure of any amyloid fibril is yet available. However all amyloid fibrils and many of their precursor molecules have a rich content of β-sheet secondary structure [2] and partial unfolding of precursor molecules, favoured by destabilising single residue substitutions in the case of hereditary amyloid, may expose aggregation-prone sequences, possibly β-strands or sheets, that promote stacking of globular subunits into amyloid fibrils [87, 88]. The three dimensional structure of apoAI is not known but the *N*-terminal region that comprises the fibril subunit in apoAI amyloidosis is predicted to adopt a predominantly β-sheet conformation [89]. It is therefore possible that the additional arginine residue in this domain, and/or the extra positive charge it confers may mediate a pro-amyloidogenic destabilisation or unfolding of the variant apoAI. The presence in the amyloid fibril subunit of methionine sulphoxide at residue 86 may be evidence

of such unfolding since it has been reported that Met86 in wild type apoAI is not oxidised following chromatographic purification, and may even be resistant to artificial oxidation, compared to Met112 and Met148 that are always oxidised to some extent [90].

Alternatively the processing and proteolytic degradation of the Arg variant apoAI molecules may differ from those of wild type apoAI, leading to production of an abnormal, amyloidogenic, *N*-terminal fragment. The physiological cleavage pathway of apoAI is not known so it is not clear whether the *N*-terminal fragments, from residues 1-86 up to 1-94, that are found in the amyloid fibrils, are normally generated from wild type apoAI *in vivo*. However it is of interest that similar fragments have been identified in all apoAI fibrils that have been adequately characterised, regardless of the Arg substitution position, 26, 50 or 60. In the original Gly26Arg variant, the sequence up to residue 83 was obtained but the intact fibril subunit was not studied, nor was the precise *C*-terminal determined [42]. In the Leu60Arg variant [19] we demonstrated fragments corresponding to residues 1-88, 92, 93 and 94, compared to 1-86, 92 and 93 in the present case.

The isolated splenic amyloid fibrils from the Leu60Arg patient [19] also contained traces of apoAI migrating in SDS-PAGE at about 28 kDa, the same position as whole intact mature apoAI. Insufficient material was available to identify this as wild type or variant apoAI, but its presence raises the question of whether cleavage of the *N*-terminal fragment is a pre- or post-fibrillogenic event. Although proteolytic cleavage has been considered a necessary precondition for deposition of precursor molecules as amyloid fibrils, and this may be true for some proteins, others such as human β2-M [38], human transthyretin [37] human lysozyme [21] and duck serum amyloid A protein [91] form fibrils without any cleavage. Furthermore recent work indicates that mouse serum amyloid A protein, which like apoAI is an apolipoprotein of high density lipoprotein, undergoes cleavage to amyloid A protein (AA) the fibril subunit of reactive amyloidosis, after rather than before accumulation at sites of AA amyloid deposition [92, 93]. It is thus of interest, firstly, that the plasma clearance rate of apoAI Gly26Arg, reconstituted into high density lipoprotein particles, was found to be more rapid than that of wild type apoAI both in normals and in individual Gly26Arg

heterozygotes, and secondly, that there was extravascular sequestration and delayed catabolism of the variant apoAI compared to normal [94].

Only variant apoAI molecules have been associated with amyloidosis in man, but the pulmonary vascular amyloid deposits that occur commonly in aged dogs consist of the *N*-terminal 71-80 residues of wild type canine apoAI [89]. There are 11 amino acid differences between human and dog apoAI up to residue 80, and 8 of these are either moderately or extremely conservative substitutions. The only 2 major changes are insertion of an extra proline residue in the human protein at position 3, and replacement of the polar serine residue at position 30 in human apoAI by hydrophobic alanine in dog apoAI.

Whatever the mechanism by which arginine substitutions, or the other differences between dog and human apoAI, are amyloidogenic *in vivo*, the present observations enhance the potential value of this system as a model for investigation by protein engineering. Studies are currently in progress of the effects of arginine substitutions at different positions, and of other mutations causing charge changes, on the properties of the *N*-terminal domain of human apoAI expressed *in vitro*. In addition to effects on fibrillogenesis, these substitutions may modulate interactions with other molecules that are universally associated with amyloid fibrils *in vivo* [1, 14], including anionic glycosaminoglycans, serum amyloid P component and apolipoprotein E.

Nothing is known of the factors other than the amyloidogenic protein itself that determine where, when and how much amyloid is deposited *in vivo* in any form of amyloidosis. Inheritance of the apoE4 genotype is associated with an increased risk of Alzheimer's disease [95] and with increased cerebral deposition of amyloid β-protein [96], but is not related to the development or age of onset of either acquired amyloid A protein amyloidosis or familial amyloidotic polyneuropathy caused by Met30 variant transthyretin (unpublished observations). Intriguingly, apoE4 contains two arginine residues at positions 112 and 158, and the only sequence difference from the two other common apoE isoforms, apoE2 and apoE3, is that they contain Cys/Cys and Cys/Arg respectively at these positions [97]. However, apoE is only a trace non-fibrillar constituent of amyloid deposits [98] and since nothing definite is yet known of its pathogenic role, if any, in amyloidosis, it is

premature to speculate on aspects in common between apoE4 and the amyloidogenic apoAI variants.

The mechanisms by which amyloid deposits cause disease are also quite obscure. There is usually no significant inflammation or necrosis around amyloid *in vivo* and it has generally been assumed that the deposits are not inherently toxic but merely cause damage by physically disrupting the structure and function of affected tissues [1]. However, the recent finding that amyloid fibrils formed *in vitro* from pure synthetic peptides can cause cell death in tissue culture by inducing apoptosis, may help to elucidate this issue, especially as fibrils composed of different proteins were found to have differential toxicity for different target cell types [99, 100]. If this is indeed a general mechanism by which amyloid fibrils are pathogenic *in vivo*, and if the various tissues of different individuals had varying susceptibility to the action of the same and different amyloid fibrils, then it could explain the clinical observations.

The mainstay of treatment of hereditary apoAI amyloidosis is supportive management, including organ replacement if necessary. An affected relative of our first patient with Leu60Arg apoAI disease [19] underwent successful renal transplantation 7 years ago and remains well. The Leu60Arg proband himself had his grossly amyloidotic spleen removed four years ago and remains well with little progression of organ dysfunction despite extensive visceral amyloid deposits. Another patient from a different kindred with the same Leu60Arg mutation underwent combined heart and kidney transplantation in September 1992 for end stage cardiac and renal failure, and also remains well leading a normal life [101]. More specific approaches to treatment include measures to reduce production or availability of the amyloidogenic variant apoAI protein. Liver transplantation has been extremely successful in hereditary transthyretin amyloidosis, leading to rapid disappearance of the amyloidogenic variant from the plasma, its replacement by wild type donor transthyretin, and clinical improvement in most cases [50, 102]. Although, unlike transthyretin, apoAI is produced in the intestine and elsewhere as well as in the liver [103], even partial reduction in circulating levels of the pathogenic variant should slow progression of amyloidosis. However, given the variable rate of progression of organ dysfunction in hereditary apoAI amyloidosis,

timing elective liver transplantation correctly will be almost insuperably difficult. A more realistic prospect may be the use of probucol which reliably reduces high density lipoprotein levels by up to 25% without major side effects.

Hereditary renal and systemic amyloidosis caused by a new deletion/insertion apolipoprotein AI variant in a Spanish family

Introduction

Hereditary amyloidosis is an autosomal dominant condition caused by point mutations in the genes encoding a variety of different proteins [1, 14]. These mutations produce single residue substitutions in the mature protein that lead to the whole molecule, or a proteolytic cleavage fragment, being deposited extracellularly in the tissues as insoluble amyloid fibrils that accumulate and cause organ dysfunction presenting in adult life. Mutations in the gene for β-amyloid precursor protein cause amyloid deposition that is confined to the brain, in familial Alzheimer's disease [104, 105] and in hereditary cerebral haemorrhage with amyloidosis (Dutch type) [106]. However in all other forms of hereditary amyloidosis the deposits are systemic even though the clinical presentation is usually dominated by manifestations in particular organ systems. Mutations in some genes cause very specific patterns of disease, such as hereditary cerebral haemorrhage with amyloidosis (Icelandic type), associated with variant cystatin C [107], and Finnish familial amyloidosis, associated with variants of gelsolin [108, 109]. Mutations in the genes for other amyloidogenic proteins cause varied and overlapping clinical syndromes unrelated to the protein involved, and the same mutation can produce a different phenotype in different families and even between members of the same family. This is the case in familial amyloid polyneuropathy, the commonest form of hereditary amyloidosis, which can be caused by more than 50 different point mutations in the transthyretin gene [41, 110], and in hereditary non-neuropathic amyloidosis which can be caused by mutations in the genes for apolipoprotein AI (apoAI) [19, 77, 85, 111], lysozyme [21] and fibrinogen α-chain [22, 78]. Although the most common amyloidogenic transthyretin variant, Met30Val, does not always cause disease, all the amyloidogenic mutations in other genes seem to be completely penetrant. Nevertheless there are evidently other genetic and/or

environmental factors, unrelated to the amyloid fibril protein itself, that contribute to deposition of amyloid and its clinical effects.

Apart from their intrinsic interest and clinical significance for the rare affected kindreds, the hereditary amyloidoses provide valuable models for analysing the pathogenesis of the common acquired forms of amyloidosis. The most important of these is Alzheimer's disease, the fourth most common cause of death in the Western world, and the discovery that mutations in the gene encoding β-amyloid precursor protein can cause early onset familial Alzheimer's has already revolutionised work on this condition [104, 105]. Furthermore, elucidation of the molecular basis of amyloid fibril formation, which should be facilitated by study of the amyloidogenic variant proteins, may permit rational approaches to therapy. The lysozyme mutations that cause non-neuropathic amyloidosis [21] are particularly promising because all aspects of the structure and folding of this protein are so well characterised. However apoAI offers a different but very intriguing challenge because, although its wild type crystal structure has not yet been determined, all three amyloidogenic variants contain arginine substitutions for neutral residues within the *N*-terminal region of the molecule, and it is this fragment that forms the amyloid fibrils [18, 19, 42, 111]. None of the other known apoAI variants are amyloidogenic [112]. We report here a family with hereditary non-neuropathic amyloidosis caused by a new apoAI variant, in which 12 residues in the amyloidogenic *N*-terminal fragment have been deleted and 2 residues inserted. This is the first deletion mutation in any gene associated with hereditary amyloidosis, but most importantly it produces an extra positive charge, suggesting that acquisition of such a charge in this part of the apoAI molecule, rather than substitution by any specific residue, may be the key amyloidogenic change.

Methods

Patients.

An extended kindred from Cerdanya, Catalonia, Spain, was identified with a high incidence of systemic amyloidosis presenting with slowly progressive and uniformly fatal liver involvement. The diagnosis of amyloidosis was first made in two

asymptomatic female first cousins (Figure 3.6) with abnormal liver function tests detected during routine screening at ages 60 and 55 yrs respectively, and in whom liver biopsies were then undertaken. Subsequently the male proband for the present study (Figure 3.6), in whom chronic liver disease had been diagnosed at age 40, presented at age 61 yrs with bleeding oesophageal varices and rapidly developed fatal hepatic encephalopathy.

Clinical investigations.

All clinical chemistry, plasma lipid and lipoprotein analyses and histological examinations were performed by standard procedures. Amyloid deposits were identified histologically by green birefringence in polarised light after Congo red staining [4].

Identification, isolation and characterisation of amyloid fibrils.

Fresh frozen, unfixed tissue obtained at autopsy of the proband was examined by immunohistochemical staining precisely as described previously [85, 113]. Amyloid fibrils were isolated from fragments of spleen, liver, kidney and heart by the water extraction method [8] as lately modified for very small tissue samples [10]. Their protein subunits were separated in reduced SDS-PAGE, and subjected to automated amino acid sequence analysis, as previously described [19, 83]. The subunits were also excised from the gel and digested with endopeptidase lys-C. The resulting peptides were separated by reverse phase HPLC with on-line diode array detection (Hewlett-Packard 1090M). Fractions containing aromatic residues were identified by their absorbance spectra and characterised by matrix-assisted, laser desorption, time of flight (MALDI-TOF) mass spectrometry (Lasermat, Finnigan MAT, Hemel Hempstead, Herts, UK) as described before [19]. Selected peptides were then sequenced. Amyloid fibrils isolated from the spleen were also dissolved in guanidine hydrochloride, fractionated by gel filtration, and the single main subunit peak analysed by electrospray mass spectrometry, all precisely as described previously [19].

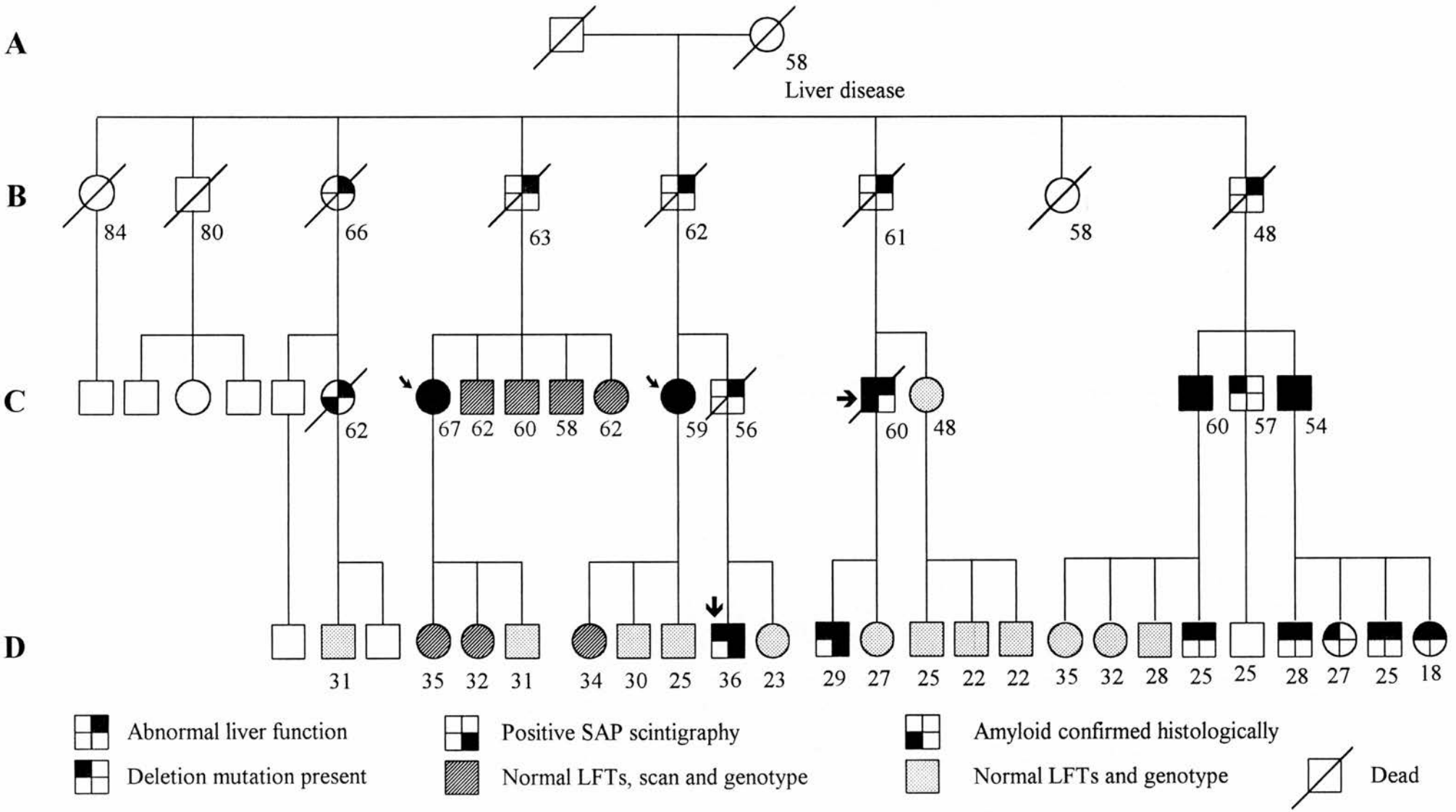

Figure 3.6 Family tree of the affected kindred. The proband is indicated by the arrow, ➔, and the two female cousins in whom amyloid was first diagnosed are indicated by arrows, ↘. The individual marked ⬇ had very low plasma apoAI and total HDL and massive hepatic amyloidosis (Figure 3.14). The age of each individual, where known, is indicated below the symbol, circles for females and squares for males. Dead individuals are indicated by a diagonal line through the symbol. The symbols are filled to show information as outlined in the legends above.

ApoAI gene studies.

DNA was extracted from the proband's blood and two fragments of the apoAI gene, comprising the region encoding the *N*-terminal region of apoAI, were amplified by PCR and the nucleotide sequence determined, all as reported elsewhere [19]. The 5′ end of exon 4 was abnormal, producing a PCR product of 361 base pairs as well as the normal 391 base pair fragment. These were clearly distinguishable in a 3% gel of low melting point agarose and this procedure was used to screen the family for other carriers. The fragments were also excised from the gel, purified by Magiprep (Promega) and sequenced [19].

Plasma apoAI analysis.

Charge variant apoAI was sought by isoelectric focusing in urea-agarose gel of delipidated whole plasma followed by pressure blotting and immunostaining with anti-apoAI antiserum, as described previously [19, 114].

Serum amyloid P component scintigraphy.

Scintigraphy after intravenous injection of radioiodinated pure human serum amyloid P component (SAP) was performed exactly as described previously [58, 59] except that the isotope ^{131}I was used instead of ^{123}I. Although the image quality is less good the longer isotope half life was necessary because the SAP had to be labelled in London and flown to Barcelona for the patient studies.

Results

Clinical features.

Affected individuals in generations A and B of the family (Figure 3.6) died with liver failure between 48-66 yrs of age. Apart from their abnormal liver function tests no other clinical or pathological information is available but it is likely that the female case in generation A was the source of the disease. Cases in generation C were found to have abnormal liver function tests at about 35-45 yrs of age but the patients all remained asymptomatic for the next 15-20 yrs. During this phase serum concentrations

of alkaline phosphatase, transaminases and γ-glutamyl transpeptidase were raised but bilirubin was normal. There were no symptoms or signs of involvement of the kidneys, peripheral or autonomic nervous system, or any other tissues or organs. Liver function slowly deteriorated in all affected cases and in the proband there was unusually rapid progression over about one year to frank cholestasis, portal hypertension, hepatic encephalopathy and death. Renal failure and proteinuria occurred during the terminal liver disease.

Percutaneous liver biopsies from four affected family members in generation C showed similar histology, with some preservation of overall structure but most of the portal tracts replaced by large amyloid nodules with some compressed arterioles and ductules at the periphery and a few foamy macrophages. Central veins and hepatic trabeculae appeared normal. A transjugular liver biopsy, from the patient who subsequently died and underwent post mortem examination, consisted of fragments of heavily amyloid laden tissue with conspicuous venous structures but no identifiable hepatocytes or ductules.

At autopsy his liver was markedly enlarged (weight 6000 gm) with a granular capsule and small focal parenchymal areas of calcification. The right lobe was completely replaced by massive amyloid deposition and the left contained large confluent amyloid nodules among areas of cholestatic hepatic tissue (Figure 3.7). The spleen was also large (1300 gm) with massive subcapsular amyloid and multiple nodular deposits within the parenchyma, unrelated to the white pulp (Figure 3.8). The kidneys were small with hard yellow nodular masses in the papillae, corresponding microscopically to diffuse interstitial amyloid deposition (Figure 3.9) limited to the medulla and associated with tubular atrophy. The renal cortex contained no amyloid; there was moderate atherosclerosis in the glomeruli and vessels. The heart was slightly enlarged and contained extensive interstitial amyloid (Figure 3.10) but myocardial fibres were preserved. The adrenals were enlarged, right 60 gm and left 35 gm, and massively infiltrated with amyloid leaving only minimal islands of cortical cells. Both testes were also massively replaced with amyloid and showed marked atrophy of seminiferous tubules. There were perivenular amyloid deposits in the pituitary gland and its capsule

and focal deposits were present in the thyroid, prostate, periprostatic sympathetic ganglia, bone and articular cartilage. The lamina propria of the tongue contained a band-like amyloid deposit but the rest of the gut was spared as were the lungs, bronchi, pancreas, lymph nodes, skin and subcutaneous fat.

Identification of the amyloid fibril protein.

Immunohistochemical staining of all the amyloidotic tissues obtained at autopsy was strongly and specifically positive with antibodies to apoAI (Figure 3.8, Figure 3.9, Figure 3.10) and to SAP, but was completely negative with antibodies to amyloid A protein, κ and λ immunoglobulin light chains, transthyretin, β2-M and lysozyme.

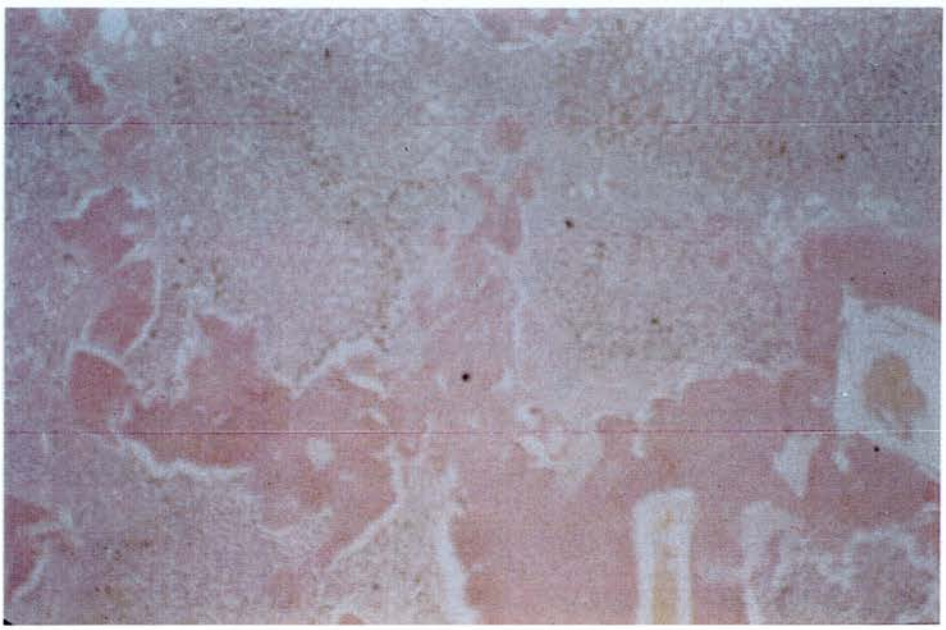

Figure 3.7 Cryostat section of unfixed liver from the autopsy of the proband, stained with Congo red and viewed in bright light, showing massive amyloid nodules among areas of cholestatic hepatic tissue. X100.

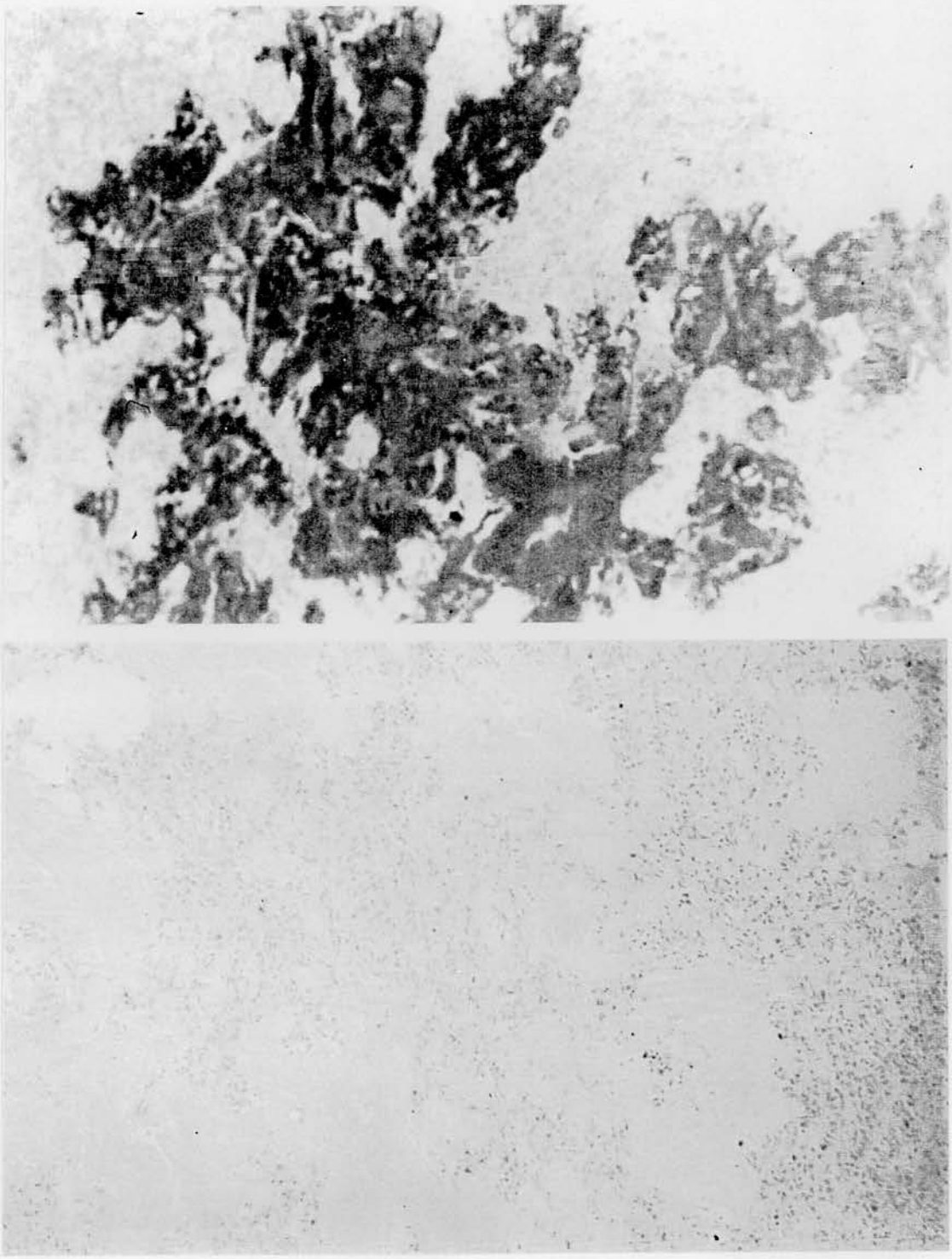

Figure 3.8 Sections of fixed spleen from the autopsy of the proband, stained by the immunoperoxidase technique with antiserum to apoAI. *Top.* Strong positive staining of the massive amyloid deposits with unabsorbed antiserum; *Bottom.* Complete absence of staining with antiserum to apoAI that had been absorbed with pure human HDL. X100.

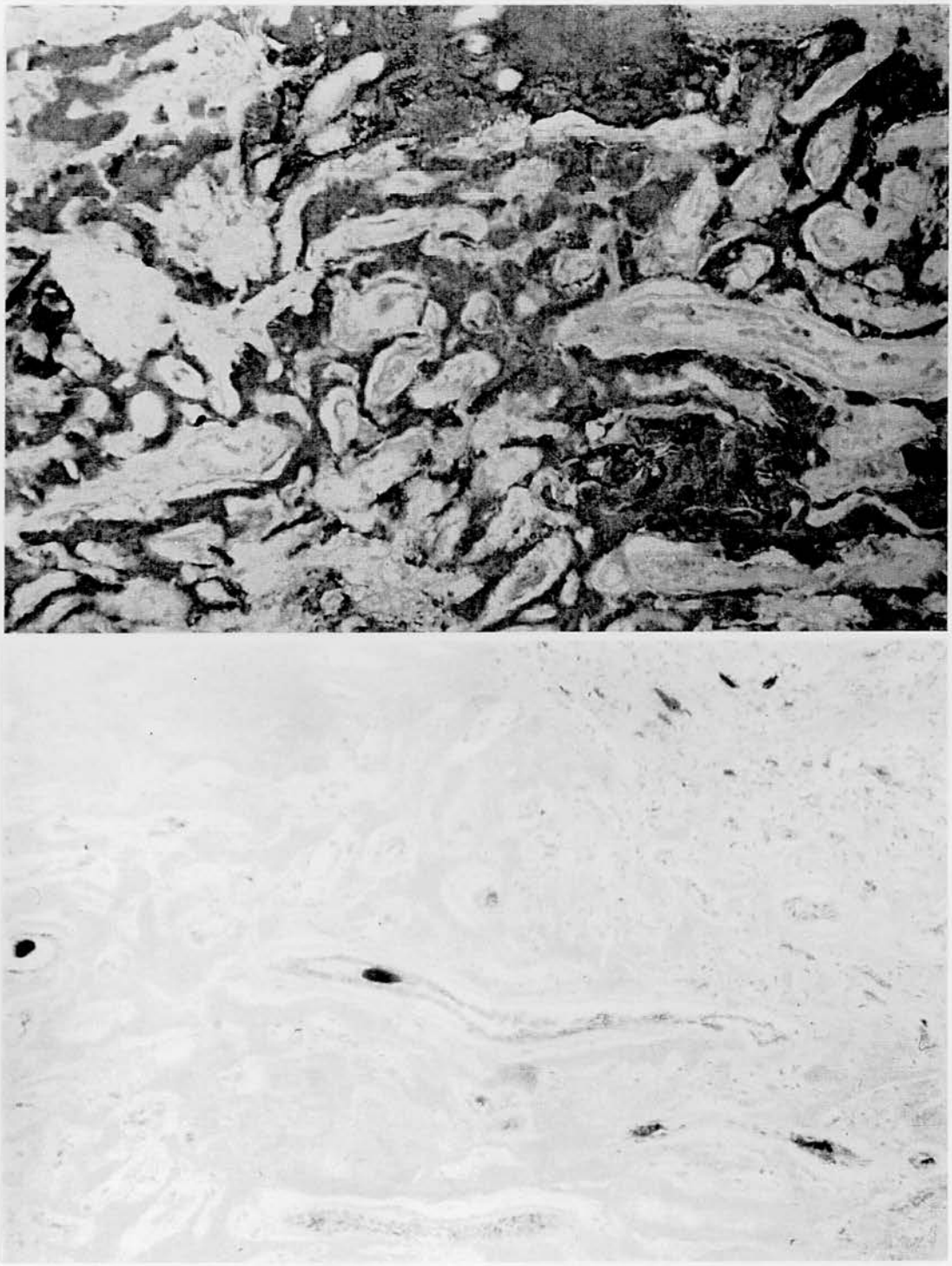

Figure 3.9 Sections of fixed kidney from the autopsy of the proband, stained by the immunoperoxidase technique with antiserum to apoAI. *Top.* Strong positive staining of the massive interstitial amyloid deposits with unabsorbed antiserum; *Bottom.* Complete absence of staining with antiserum to apoAI that had been absorbed with pure human HDL. *Renal deposits were confined exclusively to the interstitium with sparing of the glomeruli.* X100.

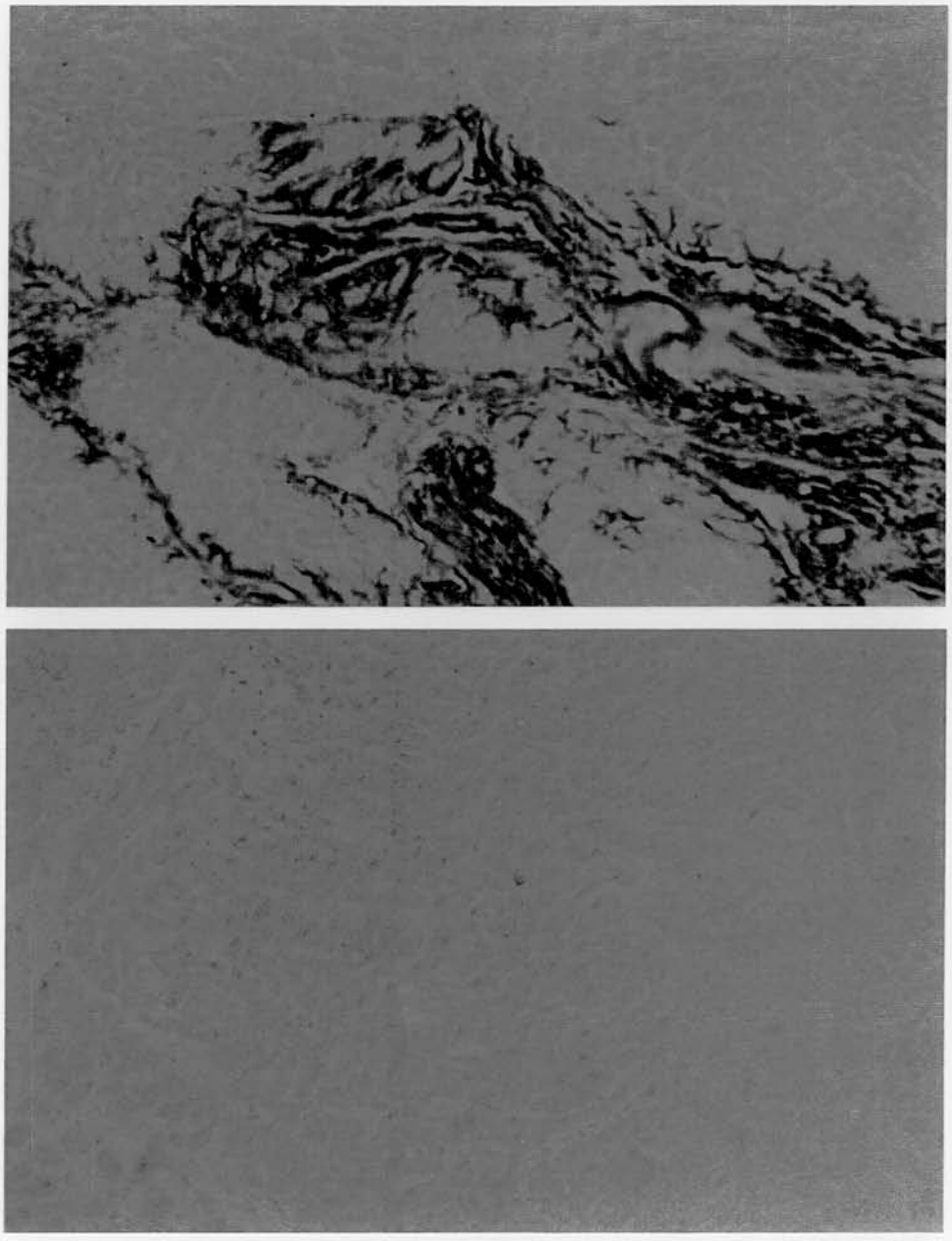

Figure 3.10 Sections of fixed heart from the autopsy of the proband, stained by the immunoperoxidase technique with antiserum to apoAI. *Top.* Strong positive staining of the massive amyloid deposits with unabsorbed antiserum; *Bottom.* Complete absence of staining with antiserum to apoAI that had been absorbed with pure human HDL. X100.

Characterisation of the apoAI gene mutation.

Sequencing of the apoAI gene from the deceased patient revealed that he was heterozygous for a mutation in exon 4 in which 35 nucleotides had been deleted from the wild type sequence and 5 nucleotides inserted, maintaining the reading frame (Figure 3.11). The mutation was readily demonstrable by gel analysis of the exon 4 PCR product (Figure 3.12), and this test was used to screen all members of the family from whom samples could be obtained. The observed DNA changes encoded deletion of residues 60-71 of the mature wild type amino acid sequence and their replacement by two new residues, ValThr. This predicted acquisition by the variant apoAI molecule of one additional positive charge, compared to wild type apoAI.

Plasma lipid and lipoprotein studies.

Among the family members from whom results were available the plasma levels of apoAI and HDL were significantly lower in carriers of the mutation than in unaffected individuals (Table 3.4), and were also lower than the normal range. There was a trend towards higher levels of LDL among the cases, although this did not achieve statistical significance, but the values for apoB, total cholesterol and triglycerides were the same in the two groups. Subsequent measurements of plasma lipids and lipoproteins in other laboratories showed exactly the same pattern in further carriers and unaffected family members (data not shown).

Variant apoAI with one extra positive charge was detected in the plasma of carriers of the mutation, but was significantly less abundant than wild type apoAI, especially in one individual with massive amyloidosis and a particularly low concentration of HDL and total apoAI (approximately 50% of the lower limit of normal). The variant was not detected in unaffected family members.

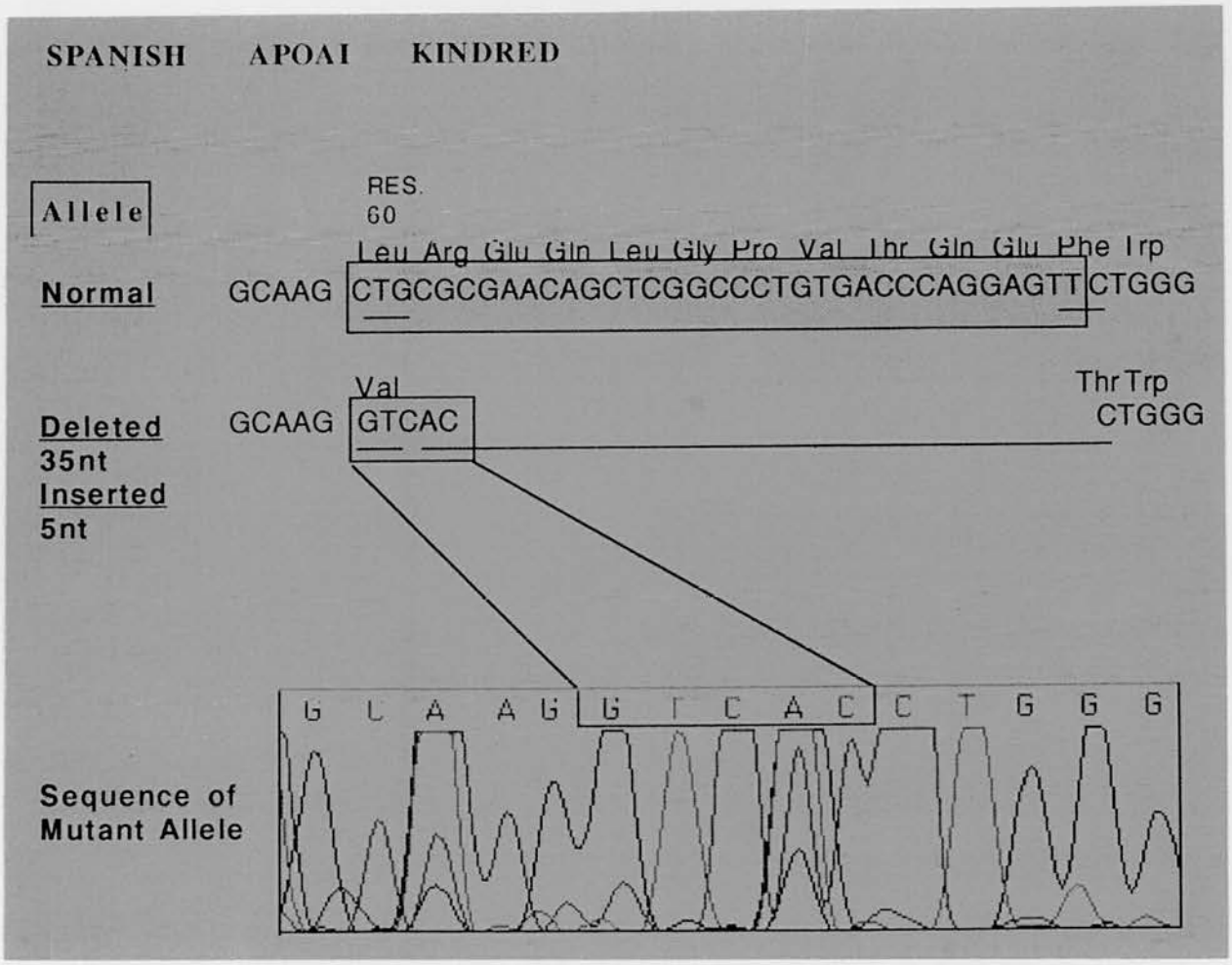

Figure 3.11 Nucleotide sequence of part of exon 4 of the apoAI gene from the proband, showing the deletion and insertion mutation.

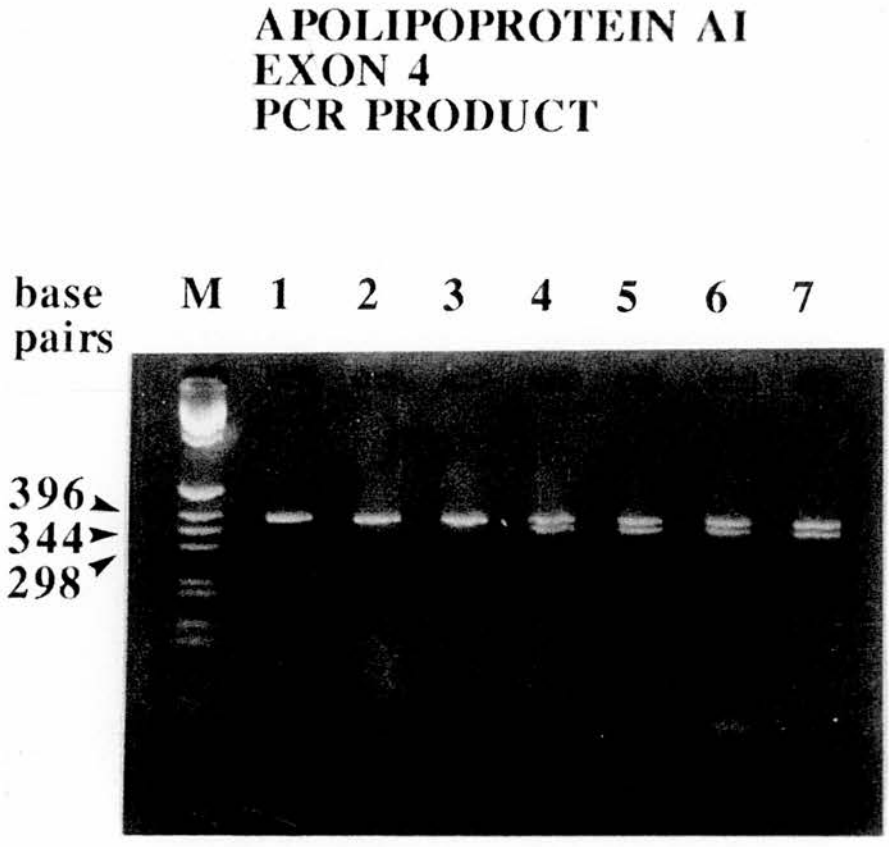

Figure 3.12 Agarose gel electrophoresis of the PCR product from the 5′ end of exon 4 of the apoAI gene. Affected individuals (lanes 4-7) yielded a product of 361 base pairs in addition to the normal 391 base pair fragment seen in unaffected family members (lanes 1-3). Markers of known length were run in lane M.

Table 3.4 Plasma Lipid and Lipoprotein Levels in Relation to the ApoAI Gene Mutation

	HDL cholesterol	ApoAI	LDL cholesterol	ApoB	Total cholesterol	Total triglycerides
Carriers						
n	11	11	11	11	13	13
Mean	28	90	172	137	212	109
SD	14	23	45	31	61	64
Range	12-48	57-118	93-240	77-171	106-315	54-301
	$P<0.001$	$P<0.001$	NS	NS	NS	NS
Controls						
n	11	11	11	11	14	14
Mean	67	164	136	130	222	106
SD	15	42	47	38	50	57
Range	49-98	59-217	68-215	70-198	149-322	58-259

The carriers tested comprised 3 females and 10 males, mean age 41.9 yrs, SD 17.6, range 18-67; the controls were 8 female and 6 male family members, mean age 36.1 yrs, SD 12.2, range 23-60. The *P* values denote significant differences between carriers and controls; NS, not significant.

Characterisation of the amyloid fibril protein.

Amyloid fibrils isolated from spleen, liver and kidney tissue consisted predominantly of protein subunits running with apparent mass of approximately 8-9 kDa in SDS-PAGE, just ahead of the 10 kD subunit of amyloid fibrils from a case of apoAI Arg60 hereditary amyloidosis [19] (Figure 3.13). Immunoblotting (not shown) identified the subunits in the present case as fragments of apoAI and this was confirmed by automated amino acid sequencing demonstrating the amino terminal sequence of mature human apoAI.

Electrospray mass spectrometric analysis of the purified spleen amyloid fibril subunits, separated by gel filtration, identified two dominant major species and a number of trace peaks (Table 3.5). The main peaks corresponded precisely to the masses calculated for *N*-terminal fragments of mature variant apoAI encoded by the observed mutation, encompassing residues 1-83 and 1-92 respectively. The residues are numbered according to the wild type sequence but the masses are calculated to include deletion of wild type positions 60-71 and insertion of ValThr at this point. Almost all the minor peaks could be assigned to other *N*-terminal fragments of the variant sequence, and no species corresponding to fragments from wild type apoAI were seen (Table 3.5).

After endoproteinase lys-C digestion, no species corresponding to the peptide expected from wild type apoAI, LREQLGPVTQEFWDNLEK, was found by HPLC and MALDI-TOF analysis. However a species with mass 1004 D, corresponding to the peptide VTWDNLEK predicted from the sequence of the variant apoAI, was identified in each of four separate digests. This material yielded a single amino acid sequence of VTW in each case. It is not clear why sequencing was blocked after three residues, but cyclisation of the adjacent aspartic acid and asparagine residues during the Edman chemistry cycles may have been responsible.

Radioiodinated SAP scintigraphy for amyloidosis.

Scans were performed in 13 family members. Four individuals from generation C and 3 from generation D, none of whom carried the apoAI gene mutation, showed no

localisation or retention of tracer. However, all carriers of the mutation, including 4 cases with amyloid confirmed histologically on liver biopsy, had abnormal scans with uptake of tracer in the liver in each case (Figure 3.14), although there was considerable variation between patients. The weakest signal was observed in a biopsy positive 67 yr old woman who remains asymptomatic 6 yrs after discovery of abnormal liver function tests. This suggests that her amyloid load may be small and not increasing rapidly. In contrast other patients, all of whom are also asymptomatic, had much more intense uptake in the liver and occasionally also the spleen, indicating a heavy visceral amyloid load, even at the much younger ages of 29 and 36 yrs, and possibly predicting a more aggressive pattern of disease. No positive images of the kidneys, adrenals or any other organs were observed in any case, possibly because of overwhelming uptake of tracer in the massive hepatic deposits.

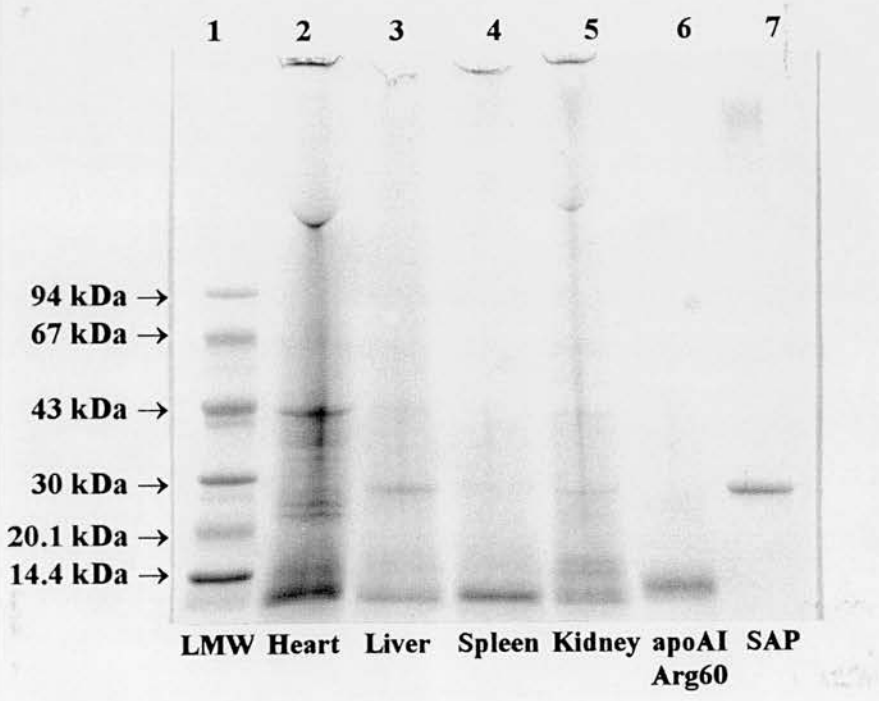

Figure 3.13 SDS 8-18% PAGE analysis of amyloid fibrils extracted from various tissues of the proband. The main band in the fibrils from heart (lane 2), liver (lane 3), spleen (lane 4) and kidney (lane 5) ran ahead of the apoAI Arg60 fibril subunit (lane 6) [19]. Marker proteins of known mass were run in lane 1 and isolated human serum amyloid P component, mass 25462 D [115], in lane 7.

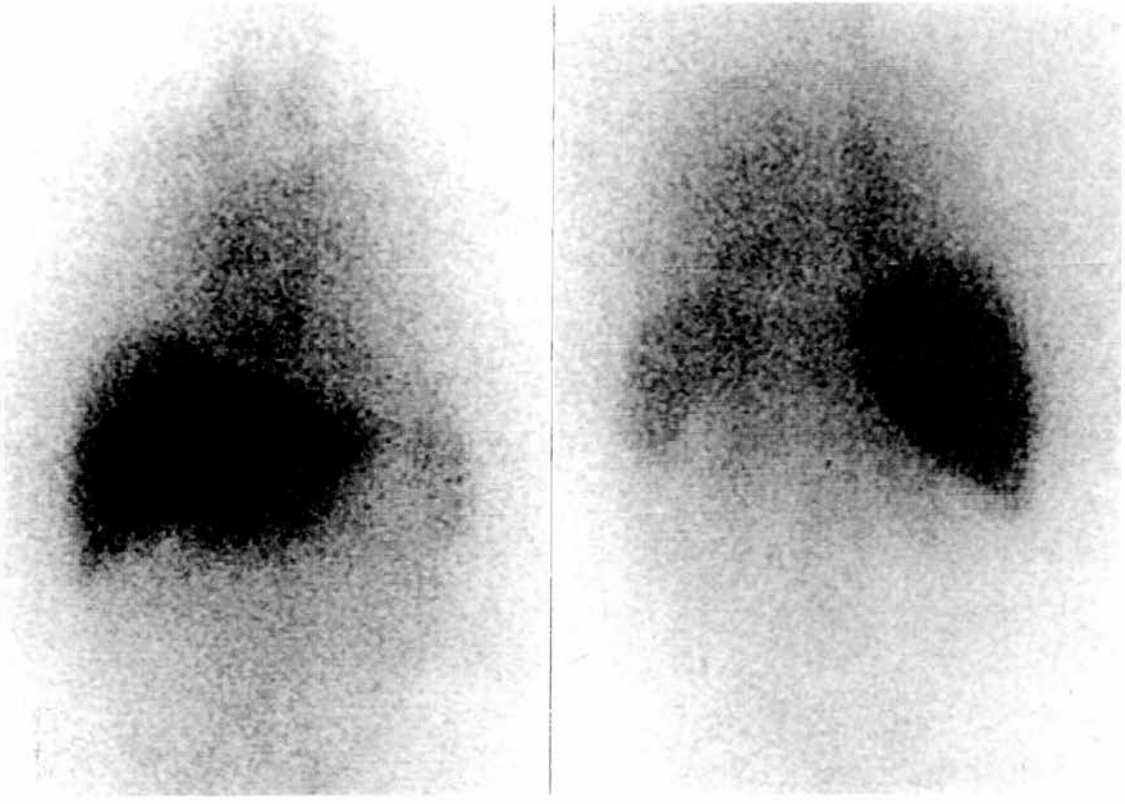

Figure 3.14 Anterior *(left)* and posterior *(right)* views of whole body scintigrams taken 24 h after intravenous injection of ^{131}I-labelled human SAP in the individual marked ↓ in Figure 3.6. There is massive uptake of tracer in the liver with no significant retention elsewhere and very low blood pool background, compatible with massive hepatic amyloidosis.

Table 3.5 Electrospray Mass Spectrometry of Purified Subunits from Splenic ApoAI Amyloid Fibrils. Measured and Calculated Molecular Masses of Predicted Fragments

	Measured mass (Da)	Predicted apoAI fragment	Calculated mass (Da)
Major peaks			
A	8332.7	1-83	8332.2
B	9438.8	1-92 including methionine sulfoxide	9438.3
Related minor peaks			
BK	8353.7	1-83 + Na^+	8355.2
BW	8373.2	1-83 + K^+	8372.2
BX	8377.4	1-83 + $2Na^+$	8378.2
BJ	9420.1	1-92	9422.2
BN	9462.1	1-92 + K^+ (or 1-92 including methionine sulfoxide + Na^+)	9462.3 (9461.3)
CA	9471.1	1-92 + $2Na^+$	9468.3
BV	9478.5	1-92 including methionine sulfoxide + K^+	9478.3
Other minor peaks			
BD	8063.7	1-81	8062.8
BT	8196.2	1-82 + Na^+	8199.0
BQ	8590.7	1-85	8589.4
BL	8638.9	1-85 + $2Na^+$	8635.4
BH	8951.9	1-88 including methionine sulfoxide	8951.8

Table 3.5 *(continued)*

	Measured mass (Da)	Predicted apoAI fragment	Calculated mass (Da)
BY	9306.5	1-91 including methionine sulfoxide	9309.2
BG	9538.4	1-93 including methionine sulfoxide	9537.4
BP	9559.4	1-93 including methionine sulfoxide + Na^+	9560.4
CC	9581.9	1-93 including methionine sulfoxide + $2Na^+$	9583.4
BO	9601.8	1-93+ $2K^+$ (or 1-93 including methionine sulfoxide + Na^+ + K^+)	9601.4 (9600.4)
BZ	9642.7	1-93+ $3K^+$ (or 1-93 including methionine sulfoxide + Na^+ + $2K^+$)	9641.4 (9640.4)
BI	10608.6	1-102 + $2Na^+$	10610.7
BE	10706.3	1-103 + Na^+	10702.7
BU′	10953.5	1-105	10955.0
CD	11582.6	1-110 including methionine sulfoxide + K^+	11582.7
BS	11965.5	1-113 + $2Na^+$	11962.2

Residues numbered according to the wild type sequence but masses calculated to include the deletion of residues 60-71 and insertion of new residues ValThr at this position. Peaks A and B were overwhelmingly dominant. The other peaks were only traces by comparison but all were assigned, as shown here, except two with masses 7644.7 and 12128.1 D respectively.

Discussion

Affected members of the family described here had a unique phenotype of hereditary, non-neuropathic systemic amyloidosis that has not been reported previously and that was caused by the first deletion mutation to be associated with amyloidosis. Although at autopsy amyloid deposits were extremely widespread, the clinical presentation, ensuing illness and death were all exclusively related to the massive hepatic involvement. In common with most other forms of systemic amyloidosis there was an asymptomatic prodromal phase, lasting for many years, with only biochemical evidence of disease. Nevertheless, liver biopsy or SAP scintigraphy demonstrated amyloid long before advent of clinical symptoms. Interestingly SAP scintigraphy suggested anticipation, at least with respect to amyloid deposition, with 3 individuals in generation D aged 29 and 36 having substantially more amyloid than 4 individuals in generation C aged between 54 and 67 yrs. Also 6 of the 7 generation D cases carrying the causative apoAI gene mutation already had abnormal liver function tests at ages between 18 and 36, whereas there was one 57 yr old carrier in generation C with no biochemical abnormality.

It remains to be seen whether clinical disease will also develop earlier and/or progress more rapidly in the youngest generation, and, if it does, they may be candidates for orthotopic liver transplantation. This would both replace organ function and reduce, though not eliminate, production of the amyloidogenic apoAI variant protein. Liver transplantation has been successful in hereditary transthyretin amyloidosis, eliminating the source of the variant amyloidogenic protein from the plasma and leading to clinical benefit in most patients and regression of amyloid deposits [50, 102, 116]. Although apoAI is produced not only by the liver but also in the intestine and elsewhere [103, 117], a significant reduction in availability of the pathogenic variant might favourably reduce the rate of amyloid deposition in a disorder that already has a very long presymptomatic phase.

The concordance between the apoAI gene mutation discovered here, the presence of the corresponding charge variant apoAI protein in the plasma, the presence of amyloid in the tissues, the identification of apoAI as the amyloid fibril protein, and

the clinical and/or biochemical signs, established the mutation as the cause of the disease. It is highly penetrant and among the 31 family members whose DNA we have been able to study, only 2 of the 13 carriers, aged 27 and 57 respectively, do not yet have any disturbance of liver function. Neither of these subjects has undergone either liver biopsy or SAP scintigraphy so the presence of amyloid deposits is not excluded.

The mechanisms by which any of the amyloidogenic variants of apoAI, including the present one, form amyloid fibrils are not known but it is unlikely to be a coincidence that they all carry one extra positive charge on the *N*-terminal part of the molecule and that the fibrils in all cases which have been analysed are composed of this fragment. In amyloid fibrils of all types the protein subunits are arranged in anti-parallel β-sheets lying with their long axes perpendicular to the fibril long axis [3, 118]. Some intact native globular proteins, some fragments of larger proteins, and some synthetic peptides have a high propensity to aggregate and adopt this conformation, that is they are amyloidogenic *in vivo* or *in vitro* [1]. The point mutations that are responsible for the autosomal dominant hereditary amyloidoses evidently increase the amyloidogenicity of the affected proteins. In the case of lysozyme, gelsolin, fibrinogen and cystatin C, the wild type molecule is not known to be amyloidogenic and either the whole or a fragment of the variant forms the fibrils. In the case of transthyretin and β-amyloid precursor protein the wild type molecule can be amyloidogenic in elderly individuals but this is greatly enhanced for the variants. Following the finding that wild type apoAI causes senile pulmonary vascular amyloidosis in dogs [89, 119] it has lately been discovered that the frequent small amyloid deposits in the aortic wall of elderly human subjects, associated with atheromatous plaques, are also derived from wild type apoAI [120].

ApoAI is thus an inherently amyloidogenic protein and this has been confirmed by the formation of amyloid fibrils from whole isolated apoAI *in vitro* [121], although *in vivo* the apoAI fibril subunits consist of *N*-terminal fragments. The variants may have enhanced amyloidogenicity because the substitutions destabilise the native structure, promoting fibrillogenic aggregation with proteolytic cleavage as a secondary phenomenon, or they may facilitate proteolytic cleavage of the native molecule to yield

fibrillogenic fragments. Also all amyloid fibrils are tightly associated with polyanionic sulphated glycosaminoglycans *in vivo* [34, 122], that are thought to contribute to formation and/or stabilisation of fibrils, and these interactions could be enhanced by the cationic nature of the amyloidogenic apoAI variants. Interestingly a synthetic peptide corresponding to residues 9-20 of the wild type apoAI sequence was amyloidogenic *in vitro* [120], although this does not necessarily relate to fibrillogenesis by the much larger fragments found *in vivo*. In the original case of Arg26 variant apoAI (Iowa) the main fragment consisted of residues 1-83 [42], whilst in human senile aortic amyloid it was 1-69 [120] and in senile dogs 1-71 and 1-80 [89, 119], all determined only by amino acid sequencing. In our electrospray mass spectrometry study of the Arg60 variant we found fragments 1-88, 92, 93 and 94 [19]. In the present case overwhelmingly the most abundant fragments were from position 1 to the residues corresponding to positions 83 and 92 in the wild type but including the deletion/insertion substitution that we have described here. In addition we detected traces of fragments 1-81, 82, 85, 88, 91, 93 102, 103, 105, 110 and 113. This level of precision cannot be achieved by sequencing without mass spectrometry, and it is likely that similar *C*-terminal raggedness and heterogeneity is present in all cases. Either there is no particular, specifically amyloidogenic, cleavage point or else the initial cleavage and/or subsequent *C*-terminal clipping proceed as post-fibrillogenic events [92].

Mass spectrometry also identified the frequent presence of sulfoxidation of Met86 in apoAI fibril subunits, both in the present case and in the Arg60 individual [19]. Whilst this might be an *in vitro* artefact generated during fibril isolation and protein purification, there is evidence that Met86 is relatively much more resistant to oxidation *in vivo* and *in vitro* than Met112 and Met148 in wild type apoAI [90]. The amyloidogenic substitutions, or in the present case deletion/insertion, *N*-terminal to Met86 might affect this property and thereby influence cleavage of apoAI and/or its association with HDL [123]. Oxidation of apoAI can also enhance its polymerisation [124].

In affected members of the present family, as in both the original Arg26 cases [18, 94] and the first Arg60 kindred [19], the plasma contained the expected charge

variant form of apoAI but at a significantly lower abundance than the wild type protein in all individuals tested. This is compatible with the accelerated plasma clearance of the variant directly documented for apoAI Arg26 by Rader et al [94], who also showed that clearance of normal apoAI was accelerated in carriers of this mutation who had subnormal plasma apoAI and HDL levels. However after plasma clearance, the extravascular catabolism of variant apoAI was significantly slower than that of the wild type, indicating sequestration, possibly in the amyloid deposits [94]. Carriers in the Spanish family studied here also had subnormal plasma levels of both total HDL and of apoAI, and the individual with the lowest plasma concentration of variant compared to wild type apoAI had massive hepatic and other amyloid deposits.

Identification of this new amyloidogenic variant extends the potential value of the apoAI model for studies of amyloidogenesis and enables informed testing and genetic counselling to be conducted in the affected family. Work on *in vitro* fibrillogenesis by apoAI is in progress and in future the effects of liver transplantation on survival, plasma apoAI levels and metabolism, and the natural history of extra-hepatic amyloid deposits will be of considerable interest.

Hereditary renal amyloidosis due to variant apolipoprotein AI Gly26Arg in an Irish family

Case Report

The proband who originates from Ireland, was 38 years old when he presented with hypertension and renal failure. He remained well until 6 years later when investigations for abnormal liver function tests (LFTs) demonstrated hepatic amyloid. There was no glomerular amyloid in his tiny renal biopsy but interstitial involvement could not be excluded. Enquiries revealed a strong family history of amyloid and renal failure. His brother died aged 47 years from colonic carcinoma but prior to that had hypertension, renal failure and abnormal LFTs with amyloid identified on liver biopsy. Their father had died also aged 47 years of unknown cause and one of his brothers had died in his early fifties of renal failure. Thirteen years after presentation, peritoneal dialysis was commenced for his renal failure and two years later, he remains alive and well with no further deterioration in liver function and no evidence for significant extra-renal disease progression.

Investigations

Methods and Results

Nodular amyloid deposits clustered around portal tracts of the proband's liver biopsy was identified on Congo red staining [4] and characterised by immunohistochemical studies using antibodies directed against serum amyloid A, immunoglobulin light chains, transthyretin, apoAI, lysozyme and β2-M as previously described [19]. Specific staining of nodular deposits were seen only with rabbit anti-human anti-apoAI antibodies and absorption control was negative (Figure 3.15), indicating staining to be specific. A subcutaneous cube of fat was taken from the proband but was not sufficiently amyloid-laden for successful fibril extraction.

Direct dsDNA sequencing of PCR amplified fragments of DNA extracted from proband's white blood cells [19] demonstrated that he was heterozygous for a single base substitution in exon 3 of the apoAI gene (Figure 3.16), changing the codon for residue 26 of the mature protein from GGC (Gly) to CGC (Arg). The rest of the sequence was normal. This mutation had previously been reported in three other families, one of whom had familial amyloid polyneuropathy with extensive visceral involvement [18, 42, 84] whilst in the other two, phenotype was of Ostertag-type with visceral amyloidosis without neuropathy [77, 85].

^{123}I-labelled SAP component scintigraphy [58-60] demonstrated a moderately large total body amyloid load with deposits in spleen and especially in the liver (Figure 3.17). Follow-up scans over the next two years remained essentially unchanged with no significant extra-renal disease progression. Abnormal LFTs, consisting of raised alkaline phosphatase also remained unchanged at follow-up.

Conclusion

The clinical phenotype, strong family history of renal failure and systemic amyloidosis in this Irish family was broadly similar to two other families with HRA [77, 85] due to apoAI Arg26 mutation. That this family has a similar genotype-phenotype relationship was supported by the demonstration of a similar mutation and apoAI in amyloid deposits. This is therefore the third family to have HRA due to variant apoAI Arg26, but the fourth family with this mutation. The original apoAI Arg26 family [18, 42, 84] had a mixed neuropathic-nephropathic presentation with disease confirmed in 8 cases on post-mortem studies. No further families with such mixed neuropathic-nephropathic disease due to any protein variants has since been reported.

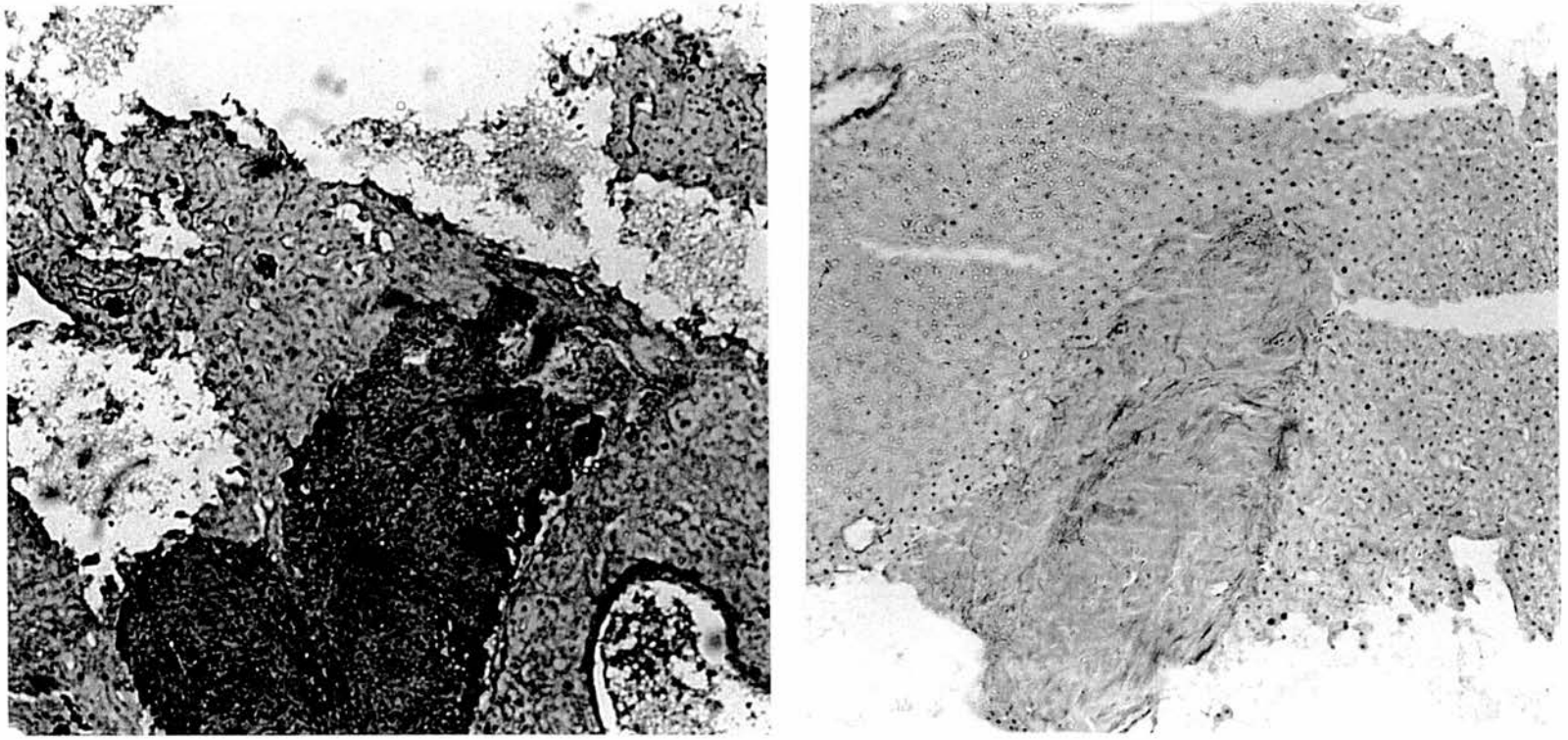

Figure 3.15 Sections of fixed liver biopsy of the proband, stained by the immunoperoxidase technique with antiserum to apoAI. *Left.* Strong positive staining of the nodular amyloid deposits with unabsorbed antiserum; *Right.* Complete absence of staining with antiserum to apoAI that had been absorbed with pure human HDL. X100.

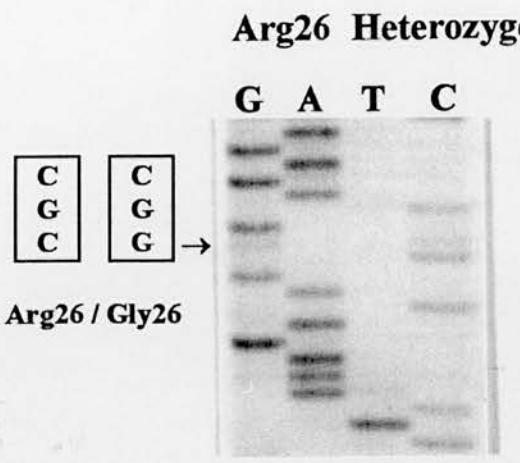

Figure 3.16 Nucleic acid sequence of part of exon 3 of the apoAI gene. The proband is a heterozygote for a single base change of G to C in codon 26.

Figure 3.17 ^{123}I-labelled SAP scan of proband 24 hours after injection of tracer. Anterior *(left)* and posterior *(right)* views demonstrating splenic and massive liver deposits.

Hereditary renal amyloidosis due to fibrinogen α-chain Glu526Val variant in a German family

Hereditary systemic amyloidosis often presents with renal disease but is rare and its significance is easily missed. Precise characterisation of the underlying metabolic defect in each kindred may have important therapeutic implications and also provides models for studying the pathogenesis of amyloidosis generally. The clinical findings and molecular basis of autosomal dominant renal amyloidosis in a large German family is described here.

Ten first degree family members (Figure 3.18) aged 23-70 years were evaluated clinically and by quantitative ^{123}I-labelled SAP scintigraphy after renal amyloid had been identified routinely in 2 cases. Scans (Figure 3.19) demonstrated systemic amyloidosis in 7 patients, affecting the spleen in all cases and, variably, the kidneys, adrenals and liver. Clinical features (Table 4.1) were hypertension, proteinuria, nephrotic syndrome and renal failure, but the age of presentation, mode of renal disturbance and outcome were remarkably inconsistent. Furthermore, correlation with the underlying quantity of amyloid was poor. DNA sequencing (Figure 3.20), confirmed by 2 RFLP analyses, identified a missense point mutation in the fibrinogen α chain gene, coding for a previously described Glu526Val variant protein. There was complete concordance between the mutation and presence of amyloid on SAP scanning.

Since fibrinogen is produced only by hepatocytes, liver transplantation as used in hereditary transthyretin amyloidosis ("surgical gene therapy") may also be potentially curative in this disease. However in this family, progression of amyloid was slow, evidence of significant extra-renal disease was minimal and long term survival on dialysis was uncomplicated. Moreover, in one case there was no evidence of new amyloid in a renal allograft after 4 years.

This study illustrates the role of SAP scanning and molecular techniques for characterising phenotype-genotype relationships in hereditary amyloidosis. Late onset, slow evolution and modest whole-body amyloid deposition, along with results in one case, indicate that renal transplantation is the therapy of choice in this family.

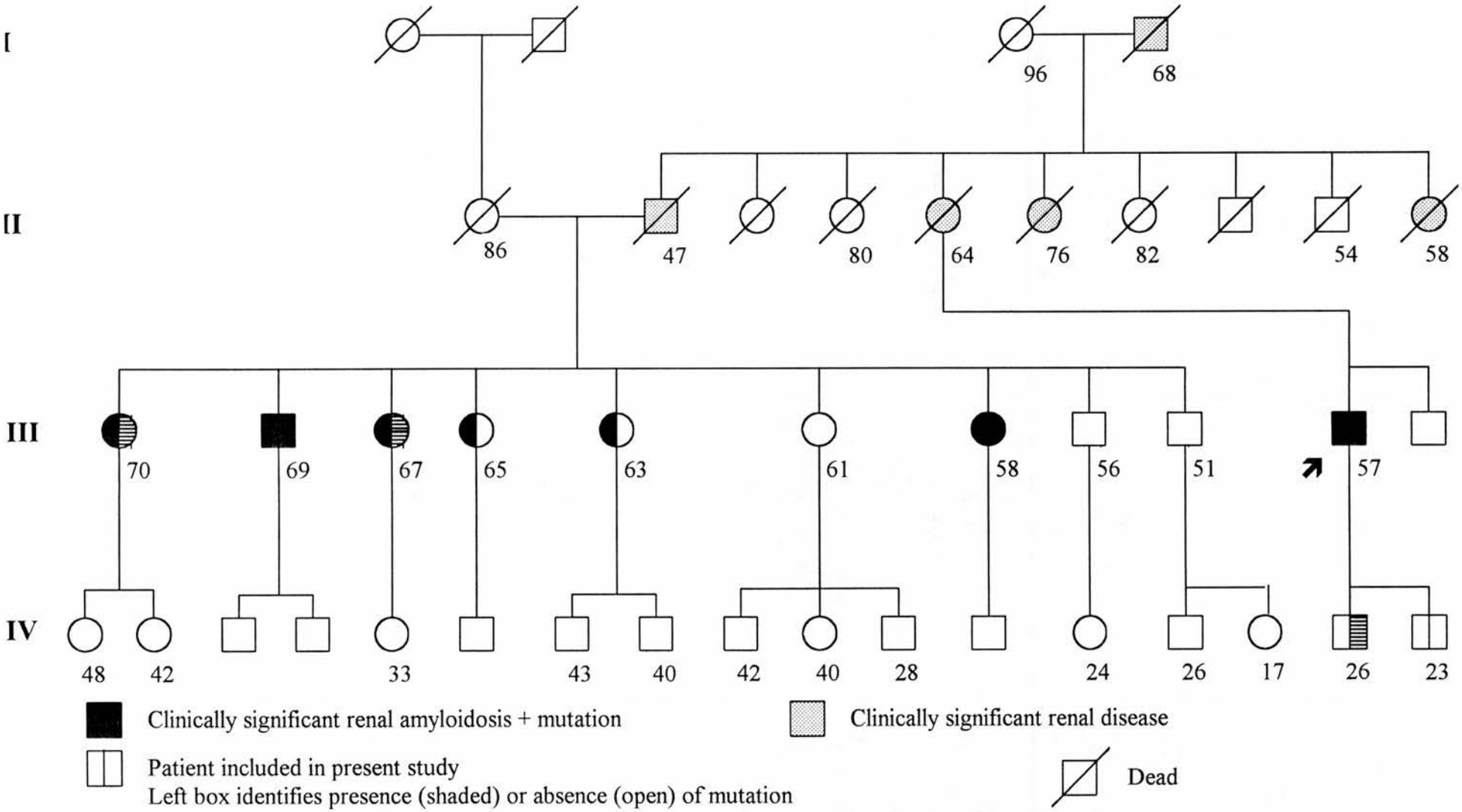

Figure 3.18 Family tree of the affected kindred. The proband is indicated by the arrow.

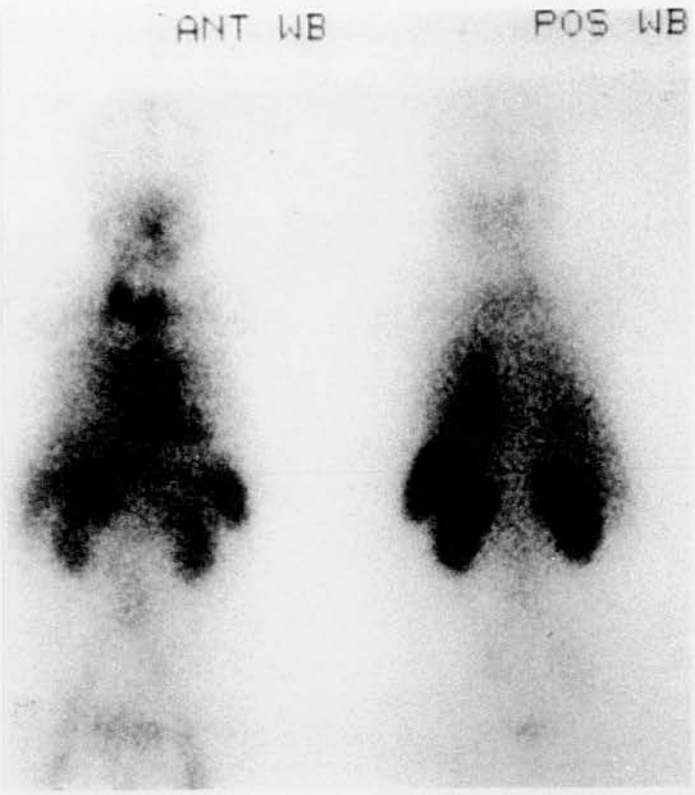

Figure 3.19 ^{123}I-labelled SAP scan of an affected individual 24 hours after injection of tracer. Anterior *(left)* and posterior *(right)* views demonstrating subclinical renal and splenic deposits.

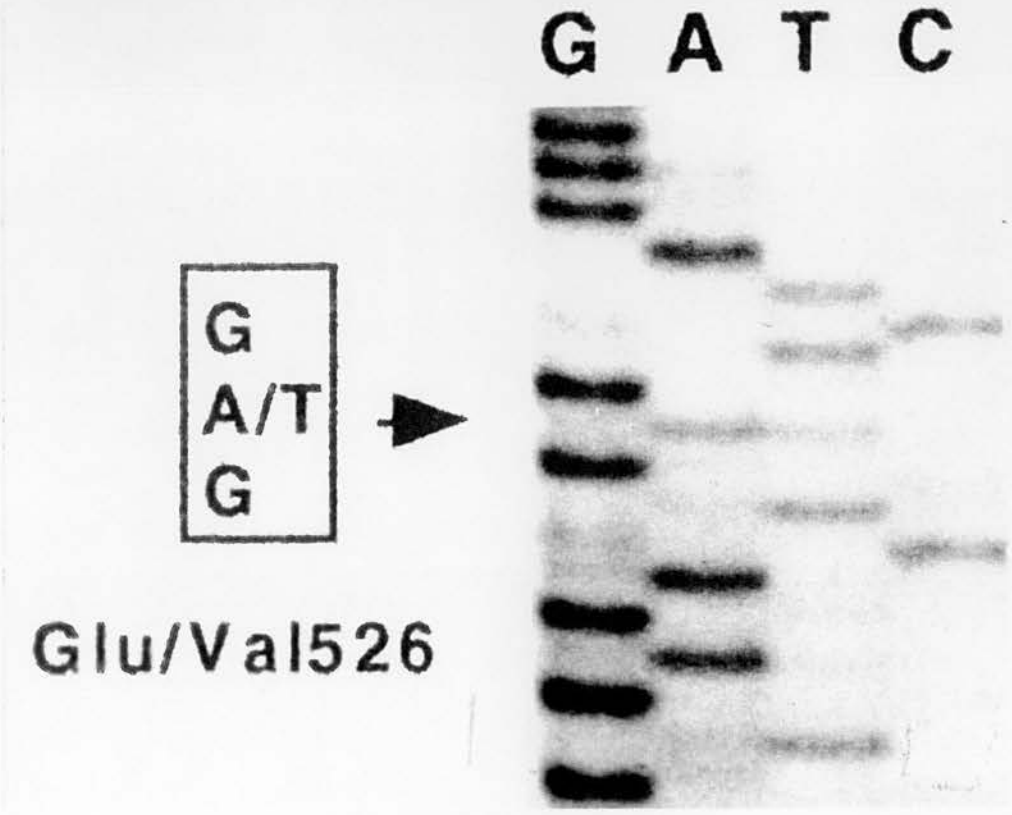

Figure 3.20 Autoradiogram of DNA sequencing gel of an affected individual demonstrating both adenine (A) and thymine (T) at position 4909 of fibrinogen α-chain indicating heterozygosity for Val526.

Chapter 4 - Clinical, scintigraphic, histological, biochemical and genetic characteristics of families with hereditary renal amyloidosis

Introduction

In 1932, Ostertag, a German pathologist, reported the first kindred with hereditary systemic amyloidosis [75]. Post-mortem examination on two brothers who died in their 30's with renal failure revealed extensive amyloid deposits in heart, kidney, spleen and liver. Since then, several kindreds have been reported to have similar extensive visceral amyloid deposits [125-128]. Pattern and severity of organ involvement may vary but these families were identifiable by the invariable involvement of the kidneys and absence of neurological disease, distinguishing them from the more common hereditary neuropathic amyloidoses, subsequently described by Andrade in 1952 [129]. Therefore the term hereditary renal amyloid (HRA) is sometimes used to describe Ostertag type families with hereditary systemic (non-neuropathic) amyloidosis. The molecular basis of this disease was first characterised only in the 1990s, and since then eight amyloidogenic variants of three circulating plasma proteins which are not normally amyloidogenic have been described [19, 21, 22, 44, 77, 78, 85, 111, 130]. The clinical, scintigraphic, histological, biochemical, and genetic characteristics of families with HRA where the disease has been fully characterised is reviewed here.

Proteins associated with hereditary renal amyloidosis

Variant apolipoprotein AI

The incidence of electrophoretic variants of apoAI is 1:1000 [86] and although more than 20 amino acid substitutions together with 2 frame shift

mutations and one inversion have now been identified, only a few are pathogenic causing abnormal cholesterol metabolism, premature coronary artery disease (especially in homozygotes where apoAI may be completely absent from the plasma compartment) or, hereditary systemic amyloidosis [112]. In heterozygotes with variant amyloidogenic apoAI, the circulating levels of both variant and wild-type apoAI [19, 44, 85, 111] are low and could be due to accelerated catabolism of variant and to a lesser extent wild-type apoAI [43]. Interestingly, all 3 amyloidogenic apoAI point mutations result in the substitution of a neutral residue by the positively charged arginine, and the more complex deletion mutation, also results in the acquisition of an extra positive charge in variant apoAI, suggesting that this structural change rather than a specific residue substitution plays an important role in amyloidogenesis, perhaps by mediating a pro-amyloidogenic destabilisation or unfolding of the aorta. The presence in the amyloid fibril subunit may be evidence of such unfolding, since it has been reported that Met86 in wild-type apoAI is not oxidised following chromatographic purification, and may even be resistant to artificial oxidation, compared to Met112 and Met 148 that are always oxidised to some extent [90].

Kindreds with amyloidogenic apoAI variants typically have extensive visceral amyloid load affecting heart, adrenals, gut and especially liver, spleen and kidneys. Most present with hypertension, proteinuria, haematuria, chronic renal failure, and hepatosplenomegaly. The disease has an autosomal dominant pattern of inheritance and penetrance appears to be complete in all cases studied so far. However, the disease which may present as early as the third decade may vary in its course even amongst members of the same kindred, although it always terminate in end-stage renal failure, necessitating renal replacement therapy with dialysis or renal transplantation. Clinical features are therefore very similar to the original description of the disease by Ostertag in 1932. The exception is the Spanish family with the apoAI deletion mutation where affected individuals presented with liver disease and death was from liver failure whilst renal function remained well preserved despite extensive interstitial amyloid [44].

Histologically, the variants may be divided into 2 smaller groups according to the pattern of distribution of renal involvement by amyloid. In kindreds with the

deletion mutation (Figure 3.8) and Arg26 variant, only renal interstitium was infiltrated with amyloid and in contrast, with the Arg60 (Figure 4.1) and especially Arg50 variant, only the glomeruli were severely affected, similar to that reported in the Ostertag brothers. The reasons for localisation of deposits to glomeruli or interstitium are not obvious although it is interesting that other forms of systemic amyloidosis also usually affect only the glomeruli (Figure 4.2) [131, 132].

Variant lysozyme

Lysozyme is a ubiquitous bacteriolytic enzyme present in external secretions, polymorphs and macrophages [133], but its physiological role has not been fully defined [134]. The first lysozyme variants known to cause disease in man, Thr56 and His67, were reported recently in two unrelated English families with hereditary amyloidosis [21]. Amyloid fibrils extracted from the kidney of the proband with lysozyme Thr56 mutation were composed of the full length variant lysozyme molecules. As with other families with hereditary renal amyloidosis due to other variant proteins, the disease has an autosomal dominant pattern of inheritance and the mutation appears to be highly penetrant. Phenotype in the Thr56 family was characterised by unique presentation with petechiae, which may be obvious even in childhood [135]. In addition, two members also presented with abdominal pain resulting in laparotomy and splenectomy. Liver in both cases were grossly enlarged and almost completely replaced by amyloid but the heart does not appear to be affected in all 3 cases where post-mortem examination was performed. In the His67 family [126], hepatic involvement was not prominent with no gross hepatomegaly and amyloid deposits were confined to the portal vessels in at least 2 of 3 cases on post-mortem examination. In the kidneys, glomeruli and interstitium were infiltrated with amyloid in both families (Figure 4.3), although progression to renal failure appears to be more rapid in individuals with lysozyme His67 variant.

Whilst the role of lysozyme is not entirely clear, there is evidence that impairment of its function increases the risk of bacterial infection [136]. Preliminary results of functional studies on serum and saliva of an affected individual using the lysoplate assay as previously described [137] suggest that its physiological actions are not impaired (Pepys et al, unpublished observations), consistent with the absence

of a clinical history of increased susceptibility to bacterial infections in both kindreds where the commonest cause of death was renal failure.

Evaluation of the possible effects of variant residues on the crystal structure of wild-type lysozyme which has been resolved to 1.5Å [138] suggest that both amino acid substitutions which occur in highly conserved regions of the protein have a destabilising effect on the structure of lysozyme [21]. As wild-type lysozyme is not normally amyloidogenic, structural analysis of the present variants and lysozyme fibrils may yield novel information on the general mechanisms of amyloid fibrillogenesis.

Variant fibrinogen α-chain

Fibrinogen, a major plasma protein involved in the final phase of blood coagulation is composed of two sets of three different polypeptide chains (α, β, and γ) [139]. Several fibrinogen α-chain variants have been determined for inherited dysfibrinogenaemias and most are single amino acid substitutions affecting the 19 N-terminal residues of the mature protein [139]. None are known to be amyloidogenic. The first amyloidogenic fibrinogen variant [22], involving the α-chain (Leu554), was described in 1993 in a Peruvian-American family. Fibrils extracted from the kidney of the proband were derived exclusively from the C-terminal fragment of the variant protein. Since then, a second amyloidogenic fibrinogen variant which also affect the C-terminus of α-chain, Val526, have been identified in two unrelated Irish-American families [78] and one German family [130]. Immunohistochemical studies confirmed fibrinogen to be the precursor protein but full fibril protein characterisation was not possible in these families.

As with other families with the disease, there was variation in phenotype although individuals with Leu554 variant tend to present much earlier and with more severe organ involvement, causing end-stage renal failure in three affected members aged 24 to 36 years. All have died despite replacement of renal function with haemodialysis or cadaveric renal transplantation. In contrast, none of the current generation of affected members of the three Val526 families have died from the disease or from renal failure. After more than 8 years of haemodialysis, there

was no evidence for significant extra-renal disease progression in one, and in another transplanted 4 years ago, graft function remains preserved with no recurrence of amyloid [130]. A feature common to both Leu554 and Val526 families is the lack of significant liver involvement clinically, biochemically and histologically. In only one out of 4 cases where tissue was available, small amyloid deposits were identified within vessel walls but not liver parenchyma. Scintigraphically, deposits were confined mainly to kidneys and spleen, with minor deposits in the liver demonstrated in only 2 of the 5 cases with abnormal scans. Another common feature was the absence of significant cardiac involvement clinically, echocardiographically and histologically. In both cases where heart tissue was available, no amyloid was demonstrated. Finally in all 5 cases where the pattern of renal distribution of amyloid was described, deposits were confined to the glomeruli.

The fibrin forming potential of variant amyloidogenic fibrinogen remains intact on functional studies [78] although thrombolysis of formed clots, which is impaired resulting in recurrent thrombosis in other non-amyloidogenic fibrinogen variants, was not studied. However, in none of the four families was there a history of bleeding or clotting disorders.

Histological, scintigraphic and fibril protein characteristics associated with hereditary renal amyloidosis

Renal histology

Heterogeneity in distribution of amyloid was also apparent within the kidneys (Table 4.1, Figure 3.8, Figure 4.1, & Figure 4.3). Even within the glomeruli, deposits may be diffusely distributed with preservation of glomerular architecture or, may be nodular resulting in disruption and in some cases replacement of glomerular structure by amyloid. In other kindreds amyloid was confined to the interstitium (identifiable only on electron microscopy in one individual) [85] or, less commonly, amyloid may distributed between glomeruli and interstitium. However, within an individual, distribution was consistent although it

may conceivably change with disease progression. Similarly, the pattern of distribution tend to be consistent within the same family but may vary especially between families and the reasons for such heterogeneity are not obvious. It is tempting to speculate for example, that nodular forms of glomerular amyloid may be due to slow accumulation of amyloid around a nidus of amyloid fibrils, whilst rapid deposition of variant proteins result in diffuse distribution of amyloid. Localisation of amyloid to the interstitium is distinctly uncommon in systemic amyloidosis [131, 132] and may be due to abnormal processing of precursor protein [132]. In addition, the function of glomerulus as a filter suggest that charge and size of precursor protein may be important determinants of the pattern of distribution of amyloid between glomerulus and interstitium. Individuals with glomerular amyloid tend to have heavier proteinuria and although interstitial diseases in general are more likely to cause renal failure, it is interesting that renal function in the Spanish family with apoAI deletion mutation for example, remained well preserved despite extensive interstitial amyloid [44], confirming our previous observations that amyloid load does not necessarily correlate with organ dysfunction [13, 49].

Table 4.1 Clinical and histological features of HRA

Year (ref)	Kindred (No of cases)	Protein	Mutation	Organ involvement (by amyloid)*					Presentation	Outcome (Renal)	Age onset	Age death
				Heart	Liver	Spleen	Kidney G	Kidney I				
1932 [75]	German (2)	?	?	-	+	+	+	-	↑bp, renal failure, hepatosplenomegaly	Death after 10 years (ESRF)		35-39
1991 [77]	Scandinavian-American (2)	ApoAI	Arg26	+	+	+	-	+	↑bp, CRF	Death after 18 years (ESRF)	25	43-58
1993 [85]	British-Canadian (4)	ApoAI	Arg26	ns	+	ns	-	+	↑bp, CRF, haematuria	ESRF after up to 20 years	20-46	
1995#	Irish(2)	ApoAI	Arg26	-	+	+	nd	nd	↑bp, CRF, abn LFTs	Death or ESRF after up to 12-13 yrs	34-38	47
1992 [19]	English (5)	ApoAI	Arg60	+	+	+	+	-	↑bp, Proteinuria, CRF, massive visceral amyloid	Four alive after up to 13 years, one case 8 years post-transplant	22-30	35
1994 [111]	Ashkenazi Jew-Australian (2)	ApoAI	Arg50	-	+	+	+	-	Haematuria, Proteinuria, renal failure, hepatomegaly	ESRF and death after 11 years	35	45
1995 [44]	Spanish (14)	ApoAI	Deletion mutation	+	+	+	-	+	Abnormal LFTs, hepatomegaly, bleeding varices	Preserved renal function, death from liver failure aged 48-66 years	18-60	50-66
1993 [21]	English (3)	Lysozyme	Thr56	ns	+	+	+	+	Petechiae, hepatomegaly, acute abdomen, proteinuria	Death after 1-24 years (CRF)	20-50	34-52
1993 [21]	English (6)	Lysozyme	His67	+	+	+	+	+	Keratoconjuctivitis-sicca, ↑bp, CRF	Death after 1-16 years (ESRF)	23-45	33-49
1993 [22]	Peruvian-American (3)	Fibrinogen	Leu554	ns	-	+	+	-	Nephrotic syndrome, renal failure	Death after up to 14 years (ESRF)	24-36	24-50
1993 [78]	Irish-American (5)	Fibrinogen	Val526	-	bv	+	+	-	Proteinuria, nephrotic syndrome, ↑bp, renal failure	Both members of older generation died of ESRF, members of current generation well on dialysis	43-66	61-69
1995 [130]	German (7)	Fibrinogen	Val526	-	+	+	+	-	Proteinuria, ↑bp, renal failure	Late-onset disease with no deaths, 2 on dialysis, 1 renal transplant	54-70	

*determined histologically and/or scintigraphically; G, glomerulus; I, interstitium; +, present; -, absent; bv, blood vessels only; ns, not stated; nd, not determined; CRF, chronic renal failure; ESRF, end-stage renal failure; LFTs, liver function tests; #, unpublished

Table 4.2 Fibril protein characteristics of HRA

Reference	Variant protein	Fibril source	Fibril protein characteristics	Fibril protein mass
Jones et al [77]	ApoAI Arg26	Spleen	Derived exclusively from N-terminal fragments of variant apoAI	11 kDa on SDS-PAGE
Soutar et al [19]	ApoAI Arg60	Spleen	Derived exclusively from N-terminal fragments of variant apoAI Residues 1-88, 1-92, 1-93, 1-94	4 major components of 10779.5, 10679.8, 10193.9 and 10177.3 Da by ESMS
Pepys et al[#]	ApoAI Arg60	Heart	Derived exclusively from N-terminal fragments of variant apoAI Residues 1-92, 1-93, 1-94	3 major components of 10779.1, 10820.9 and 10907.1 Da by ESMS
Booth et al [111]	ApoAI Arg50	Liver	Derived exclusively from N-terminal fragments of variant apoAI Residues 1-86, 1-92, 1-93	3 major components of 10606.6, 10705.6 and 9902.6 Da by ESMS
Tan et al [44]	ApoAI deletion variant	Spleen	Derived exclusively from N-terminal fragments of variant apoAI Residues 1-84, 1-92	2 major components of 8332.6 and 9438.9 Da by ESMS
Pepys et al [21]	Lysozyme Thr56	Kidney	Derived exclusively from whole intact variant lysozyme molecules	14680 Da by laser desorption mass analysis of major component
Benson et al [22]	Fibrinogen α-chain Leu554	Kidney	Derived exclusively from C-terminal fragments of variant protein	Two major components of 11 kDa and 13 kDa on SDS-PAGE

#, unpublished

^{123}I-labelled SAP scintigraphy

Radiolabelled SAP scintigraphy [58-60] has been an indispensable tool in our studies on families with HRA. The visceral pattern of distribution of amyloid first determined by Ostertag on post-mortem studies can now be studied for the first time *in vivo* by scintigraphy (Figure 3.1, Figure 3.14, Figure 3.17, Figure 3.19). In addition to demonstrating variation in pattern of visceral amyloid distribution most obvious in disease caused by different amyloidogenic proteins, subclinical amyloid deposits may also be detected in asymptomatic carriers of mutant genes [19, 44, 130], providing evidence for its use as a screening procedure and for determining concordance between mutation and disease that could previously only be confirmed in such cases on biopsy. In one kindred, only 4 out of 9 biopsies was diagnostic in 8 cases where systemic amyloidosis was subsequently confirmed on post-mortem studies [84], suggesting that scintigraphy may be a more sensitive technique in the diagnosis of systemic amyloidosis. Scans may also be used to follow rate of disease progression. We have for example, recently confirmed clinical suspicion that disease progression may be slow in individuals with fibrinogen α-chain Val526 variant, with scintigraphic studies demonstrating lack of extra-renal disease progression in an individual who have been dialysed for more than 8 years and in another individual with no amyloid recurrence in a graft 4 years post-transplant [130]. We have now studied seven different families [19, 21, 44, 85, 111, 130] with six different amyloidogenic protein variants and in all cases where tissue was available, there was complete concordance between mutation and disease. Therefore radiolabelled SAP scans may be used in the diagnosis, screening and follow-up of families with HRA.

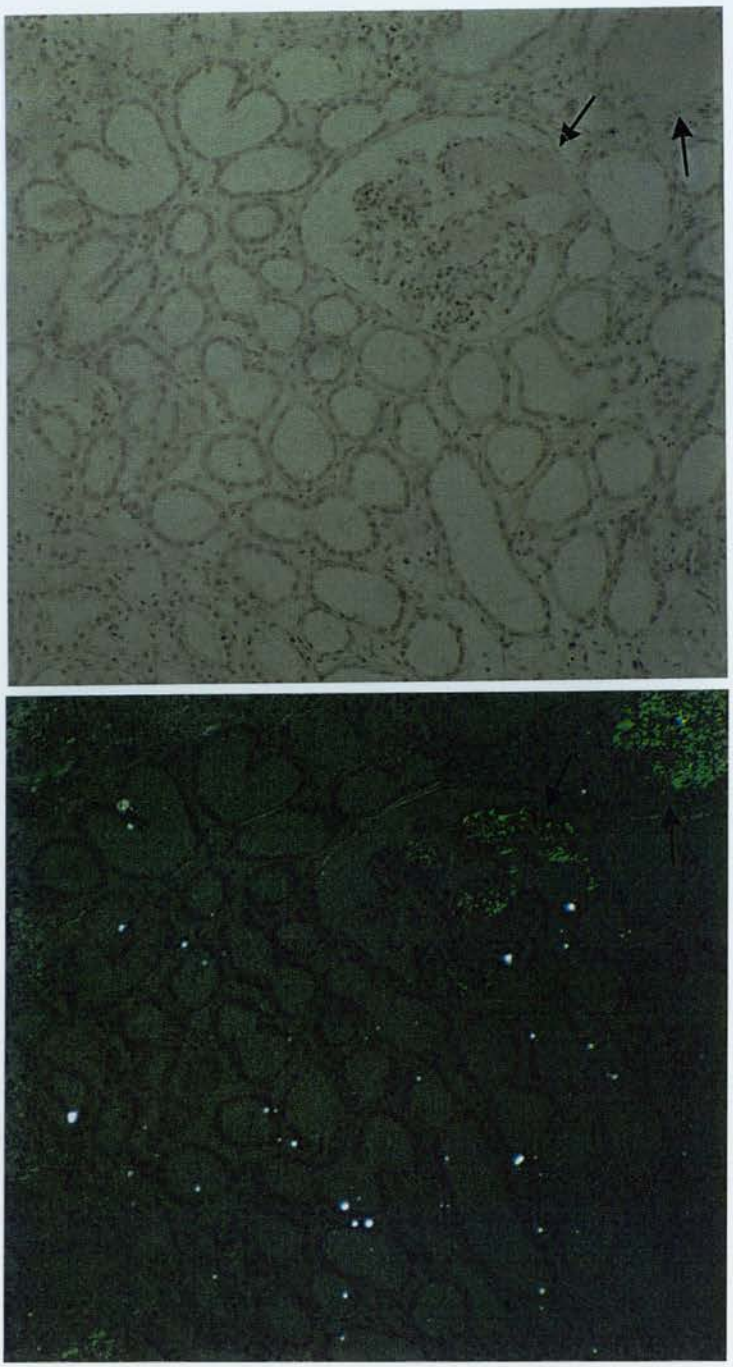

Figure 4.1 Renal section of a patient (NP) with HRA due to apoAI Arg60 [19] stained with Congo red and viewed in bright light *(top)*, and polarised light *(bottom)*, demonstrating nodular amyloid deposits *(arrow)* confined to the glomeruli. X40.

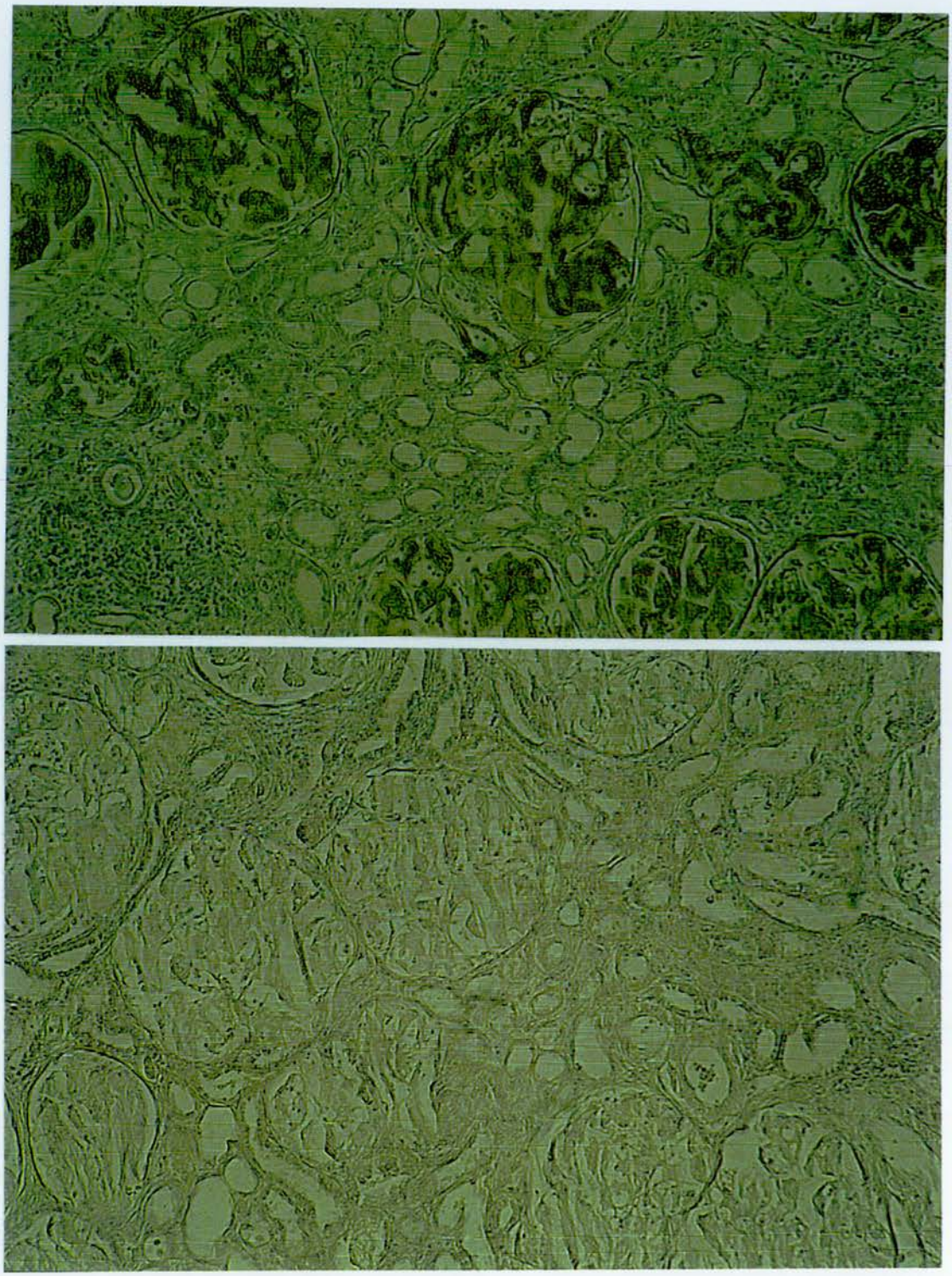

Figure 4.2 Sections of kidney stained by immunoperoxidase technique with antiserum to SAA in a case of AA amyloidosis. *Top.* Strong positive staining demonstrating diffuse amyloid deposits confined to the glomeruli with sparing of the interstitium. *Bottom.* Complete absence of staining with antiserum to SAA that had been absorbed with pure human acute phase serum. X40.

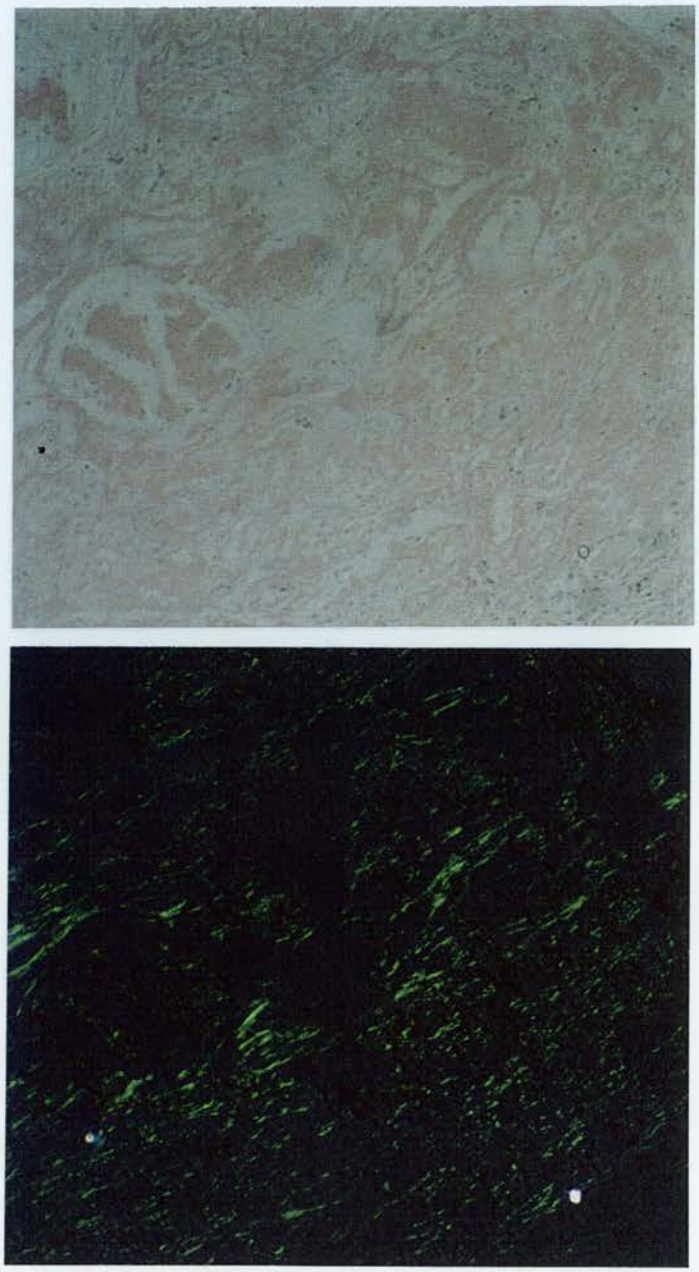

Figure 4.3 Renal section of a patient (SD) with HRA due to lysozyme His67 [21] stained with Congo red and viewed in bright light *(top)*, and polarised light *(bottom)*, showing widespread and diffuse infiltration of glomeruli and interstitium by amyloid. X40.

Fibril protein characteristics

The lack of affinity of fibril precursor protein for neural tissue is both diagnostic and striking, a unique property that is evidently preserved even in affected individuals where disease progression whilst maintained on long term dialysis might be expected to occur and involve other tissues such as nerves. The observation that this does not happen [130] will need to be confirmed and could be due to preferential localisation of circulating variant proteins into existing amyloid deposits.

In all cases where the disease has been fully characterised, the fibril precursor proteins in HRA were exclusively derived from variant molecules (Table 4.2). Characterisation of these precursor proteins may identify a common property that can provide crucial information on the mechanisms of fibrillogenesis, and may also help to explain the variation in phenotypic expression, especially between kindreds. So far, all fibril proteins are of 8 to 14 kDa in size, and in the case apoAI variants, the fibril proteins extracted from spleen, liver and heart of different kindreds are homogeneous in both size and charge. All have an extra positive charge as a result of the substitution or deletion and all are derived from the N-terminal 84 to 94 residues. Although these properties are unique and evidently critical in apoAI amyloidogenesis, it does not however lead to uniformity in phenotypic expression. Indeed, in the original apoAI Arg26 family [84], the disease had an unusual mixed neuropathic-nephropathic presentation although fibril proteins extracted from spleen and liver (sequenced up to the N-terminal 77 and 83 residues respectively) [42] were of similar size and charge.

Whilst variant apoAI and variant fibrinogen α-chain are cleaved either before or after the fibrillogenic event, this is not a necessary requirement for amyloidogenesis in general. Both wild type and variant transthyretin, wild-type β2-M and variant lysozyme may be deposited as whole intact molecules into amyloid fibrils [1, 14]. Finally, as none of the wild type proteins associated with HRA are known to be amyloidogenic, the high degree of, if not complete penetrance of the associated mutations suggest that their amyloidogenic variants may therefore serve as powerful models for studies on amyloidogenesis.

Management

The main clinical consequence of HRA is renal failure and indeed, the disease was usually fatal within 10 years of diagnosis, death being due to or related to renal failure. Supportive therapy therefore consists of replacement of renal function by dialysis or renal transplantation and this has improved the prognosis considerably [13]. For example, none of the affected members of the current generation of our German family with fibrinogen Val526 variant has died from the disease or from renal failure in contrast to earlier pre-dialysis generations where some have succumbed to renal failure in their fifth decade. Transplantation of other organs terminally affected by disease should also be considered. In December 1992, we performed a double heart and kidney transplant on a 35 year old girl with organ failure due to variant apoAI Arg60 amyloidosis and she remains alive and well 3 years later with no significant extra-renal disease progression or amyloid recurrence in her grafts (Pepys et al, unpublished observations).

Specific treatment aimed at reduction of fibril precursor protein may be accompanied by halt in disease progression and in some cases, disease regression [12, 13, 50]. Since fibrinogen is produced exclusively by hepatocytes [140], liver transplantation is potentially curative. Such surgical forms of genetic therapy have been used successfully in hereditary neuropathic amyloidosis due to variant transthyretin [50, 102], a protein that is produced almost exclusively by the liver. The synthesis of apoAI is divided approximately equally between liver and small intestine [103, 117] and therefore liver transplantation may reduce circulating variant apoAI levels sufficiently to slow down or even halt disease progression. Similar options are not available in disease due to variant lysozyme, a ubiquitous component of external secretions, polymorphs and macrophages.

As with all hereditary diseases, genetic counselling and screening form an important part of their management. Individuals who present in their early years and wish to have children may be reassured that disease progression can be slow and in

those affected prognosis may be considerably improved especially with renal supportive therapy and organ transplantation.

Discussion

Most cases of hereditary systemic amyloid either have mainly neuropathic disease which is frequently accompanied by a lesser degree of visceral organ involvement or, less commonly, it affects exclusively the visceral organs and characteristically spares the nerves [1, 14]. Since the original description of this visceral disease by Ostertag in 1932, 12 different families with amyloid due to 4 different amyloidogenic variants of apoAI [19, 44, 77, 85, 111], 2 variants of lysozyme [21] and 2 variants of fibrinogen α-chain [22, 78, 130] have been reported. Wild-type molecules of these 3 proteins are not normally amyloidogenic although wild-type apoAI may cause pulmonary amyloidosis in aged dogs [89]. The mechanisms by which these normally soluble circulating proteins are rendered amyloidogenic by single residue substitutions are obscure. All amyloid fibrils and many of the precursor molecules have a rich content of β-sheet secondary structure [2]. There is *in vitro* evidence that single residue substitutions in structurally critical positions may have a destabilising effect on protein structure, causing precursor molecules to unfold, exposing aggregation prone sequences possibly β-strands or sheets that promote the deposition of variant molecules as insoluble amyloid fibrils [87, 88]. This may be the case with variant lysozyme, where structural analysis suggest that both amino acid substitutions occur in highly conserved regions of the protein and are likely to have a destabilising effect on domain structure and stability [21].

In apoAI amyloidosis, a discernible pattern has emerged, with all variants including the deletion variant, resulting in the mature protein acquiring an extra positive charge in the N-terminal part of the molecule which is the fragment actually deposited in the tissues as amyloid (Table 4.2). The mechanism by which this renders the protein pro-amyloidogenic remains to be determined. Whilst the extra positive charge may

have a destabilising effect on protein structure, it may also possibly result in abnormal processing and proteolytic degradation of the variant molecules, leading to the production of an abnormal N-terminal fragment. Evidence for abnormal handling of variant apoAI is provided by *in vivo* studies demonstrating that plasma clearance rate of apoAI Arg26, reconstituted into high density lipoprotein particles, was found to be more rapid than that of wild type apoAI both in normals and individual Arg26 heterozygotes, and secondly, that there was extravascular sequestration and delayed catabolism of the variant apoAI compared to normal [43]. No discernible pattern has yet emerged from variant fibrinogen α-chain, the three dimensional structure of which, like apoAI, has yet to be determined. It is however significant that the Leu554 variant is amyloidogenic but not Cys554 [141] which is due to a codon change from CGT (Arg) to TGT (Cys) resulting in functional abnormalities causing thrombophilia and, although there are associated structural anomalies with abnormal fibrin formation, this variant does not appear to be amyloidogenic. Conversely, Leu554 (and Val526) variants appear to be functionally normal on clotting studies and functional assays [78]. Clearly, in addition to the site of residue substitution, the type of amino acid with changes in charges or hydrophobicity may be critical in determining phenotype. Comparison of the subtle but clearly crucial changes in biochemical and structural properties of these two variants may provide important information on the mechanisms of fibrillogenesis.

Heterogeneity was also evident in clinical phenotype with diversity in timing and distribution of amyloid, corroborated by scintigraphic and histological studies where variation of involvement was observed even within an organ, with for example predilection of amyloid deposition in glomeruli in most, whilst exclusively localised to the interstitium in some kindreds (Table 4.1). Identification of factors responsible for such diversity will clearly be of considerable clinical relevance and may improve our understanding of the pathogenesis of amyloidosis, leading perhaps to new therapeutic strategies aimed at the abnormal kinetics leading to fibril deposition.

In his follow-up paper in 1950 [76], Ostertag reported 3 other members of the family who died aged 18, 36 and 43 years, with a clinical diagnosis of amyloid, in

addition to the 2 brothers whom he previously reported their post-mortem findings. In this paper, he speculated that the amyloid, which by that time was known to be derived from proteins, was likely to be caused by a mutation associated with the protein. Descendants of this family, if any, have not yet been identified and the nature of the disease remain uncharacterised. Phenotype in the Ostertag family was distinguished by presentation with early development of hypertension and renal failure, and at autopsy, there was massive infiltration of amyloid in the clinically enlarged liver and spleen. Renal amyloid was confined to the glomeruli and the heart was not affected. This description is most closely resembled by the apoAI families, especially the Arg50 family, who originated from Poland and therefore also geographically closet to Ostertag, apart from the German fibrinogen Val526 variant family whose phenotype was however very different with minimal liver involvement and late-onset disease. The phenotype-genotype relationship in the Ostertag family may never be determined, but it is clear from the 12 families where the disease has been fully characterised that within a family, this is a monogenic disease with an autosomal dominant pattern of inheritance. The aberrant genes of which carriers are all heterozygotes, was in all families due to a point mutation encoding single residue substitutions, except for one family with the apoAI deletion mutation. The mutant genes appear to have a high degree of penetrance and phenotypically, they are identified by exclusive visceral organ involvement with invariable renal amyloid deposition but marked absence of nerve involvement. Timing, severity and distribution of other organ involvement may however, vary. Frequently fatal within 10 years of diagnosis, the prognosis has been considerably improved with renal supportive therapy, and in some, liver transplantation as a surgical form of genetic therapy may be potentially curative [1, 13].

Chapter 5 - Hereditary Transthyretin Amyloidosis

Familial amyloid polyneuropathy with predominant cardiac involvement associated with a novel variant of transthyretin, Thr59Lys, in an Italian family

Introduction

Cardiac amyloidosis is a distinct form of cardiomyopathy which usually carries a grave prognosis [142]. Amyloid deposits are largely composed of protein fibrils, the precursors of which differ in different forms of the disease, and clinical amyloidosis syndromes are classified according to the identity of these proteins and whether the disorder is acquired or hereditary [1, 11]. It is essential to characterise the underlying process in all cases since this may have major implications for prognosis and treatment. Hereditary forms of amyloidosis are rare but are extremely serious for the affected families and are also valuable models for understanding the pathogenesis of amyloid deposition in general. This is important because clinically significant amyloidosis is not rare [1]: localised amyloid deposits in the brain are a hallmark pathological feature in Alzheimer's disease and amyloid is present in the islets of Langerhans of the pancreas in most patients with Type II diabetes mellitus. Systemic forms of amyloidosis are less frequently encountered but their management is a major challenge.

Clinically significant acquired cardiac amyloidosis is usually of AL (formerly known as primary) type, in which the amyloid fibril protein is derived from monoclonal immunoglobulin light chains [1, 142]. Although so-called senile cardiac amyloid, in which normal wild-type transthyretin (TTR) is the fibril protein, is very common in the elderly, it is rarely symptomatic [142, 143]. Hereditary cardiac amyloidosis presents from the third decade onwards and usually occurs in the context of familial amyloid polyneuropathy (FAP) [142, 144], associated with peripheral and autonomic neuropathy which dominate the clinical picture, although there are families in which cardiac involvement has been the major or only clinical feature. The amyloid fibril protein in most kindreds with FAP is derived from variant TTR. In each family the

variant contains a single amino acid substitution encoded by a point mutation in the TTR gene, and more than 40 such mutations, inherited in an autosomal dominant pattern, have been identified [1, 110].

Here we report an Italian family with a new variant of TTR associated with hereditary amyloidosis and a predominantly cardiac presentation. Interestingly the clinical features in several of the affected individuals strongly suggested ischaemic heart disease. This family illustrates the diverse phenotypic expression of amyloidogenic mutations and underscores the importance of early diagnosis in cardiac amyloidosis.

Methods

Clinical Evaluation of Patients

Clinical details and the results of routine investigations were available on 7 members of an Italian family who all presented with cardiac disease. Cardiac investigation included 12-lead electrocardiography, 24 hour Holter rhythm monitoring and detailed echocardiography with Doppler analysis. Cardiac catheterisation, endomyocardial biopsies and electrophysiological studies were performed in some cases. Limited autopsies were obtained in 3 cases.

Histology

Tissue was available from each case, obtained during life in four, and at autopsy in the remainder. Myocardial tissue was studied in 6 cases, rectum in 2, and small intestine and sural nerve biopsies in 1 case each. Amyloid was identified by Congo red staining with pathognomonic green birefringence when viewed under crossed polarised light [4].

Immunohistochemical Staining

For detection of TTR, sections were first incubated overnight with 1% w/v sodium-m-periodate for 10 minutes, 0.1% w/v sodium borohydride for 10 minutes and 6 mol/L guanidine hydrochloride in 0.9% w/v NaCl, to enhance immunoreactivity. After washing with saline non-specific binding was blocked by incubation with

10% (v/v) normal non-immune goat serum in 10 mmol/L Tris-buffered saline (TBS) for 60 minutes at room temperature. Sections were then incubated overnight at 4°C with specific polyclonal rabbit anti-human TTR antibodies (Dako Ltd, High Wycombe, Bucks, UK) diluted 1:400 in TBS containing 1% (v/v) normal goat serum. Specificity of staining was established by reacting adjacent serial sections with the same dilution of antiserum previously absorbed with pure human TTR to remove all anti-TTR activity. After these primary reagents, the slides were washed on a rotating platform, twice with TBS containing Triton X-100 (BDH Laboratory Supplies, Lutterworth, Leics, UK) 0.005% (v/v) and once with TBS alone prior to incubation for 60 minutes at room temperature with polyclonal goat anti-rabbit antiserum (ICN Biochemicals Ltd, Thame, Oxon, UK) 1:50 in 5% (v/v) normal human serum. The washing as above was repeated and sections were then incubated at room temperature for 60 minutes with rabbit peroxidase-anti-peroxidase complexes (PAP) (Serotec Ltd, Kidlington, Oxon, UK) 1:50 with 1% (v/v) normal goat serum in TBS. After another wash cycle to remove unbound rabbit PAP, bound enzyme was detected using 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co Ltd, Poole, Dorset, UK) 0.05% (w/v) in TBS containing 10 mmol/L imidazole (BDH Laboratory Supplies, Lutterworth, Leics, UK), and 0.002% (v/v) H_2O_2 (Taab Laboratory Equipment Ltd, Reading, Berks, UK) as substrate. Separate sections were stained using antisera against other known amyloid fibril proteins: κ and λ immunoglobulin light chains, amyloid A protein, apolipoprotein AI and lysozyme.

Radiolabelled SAP Scintigraphy and Turnover Studies

Whole body scintigraphic imaging and a 24 hour plasma turnover study were performed in one patient (III.4) using ^{123}I-labelled serum amyloid P component (SAP), as previously described [58, 59]. Briefly, anterior and posterior whole body scans and regional images were obtained with an IGE Starcam gamma camera 24 hours after i.v. injection of ^{123}I-labelled SAP (200 MBq of activity associated with 100 μg of pure protein). The decline of radioactivity was measured in the plasma over 24 hours and

activity was also estimated in the complete collection of urine obtained for 24 hours after isotope administration.

Isolation of DNA and Amplification and Sequencing of TTR Gene

DNA was extracted from whole blood [73] and TTR exons were amplified by the polymerase chain reaction (PCR) using *taq* polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT) with the following cycling conditions: One cycle of 94°C, 5 minutes; 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and a final step of 72°C for 10 minutes. For exon 1 intron sequences, (5′ to 3′), CAGCAGGTTTGCAGTCAGAT and GGTACCCTTGCCCTAGTAAT were used, for exon 2, CAATTTTGTTAACTTCTCACG and CAGATGATGTGAGCCTCTCTC; exon 3, CCTCCATGCGTAACTTAATCC and TAGGACATTTCTGTGGTACAC; exon 4, TGGTGGAAATGGATCTGTCTG and TGGAAGGGACAATAAGGGAAT.

PCR products (100 μL) were purified by size fractionation on a Nusieve agarose gel (FMC, Rockland, ME). The band was extracted using Magiprep columns (Promega, Madison, WI), recovered by ethanol precipitation and dissolved in 12 μL of distilled water. Six μL was then used in the sequencing reaction for each primer.

The sequencing reaction was modified from Casanova et al [74]. A reaction mix containing 2 μL of sequencing buffer, 2 μL of primer (100 ng/μL) and 6 μL of template was boiled for 2 minutes, before freezing in a dry ice/methanol bath for 15 seconds, and then adding 5 μL of Mastermix. Just after the mixture thawed, 3 μL was added to 2.5 μL of each of the four dideoxynucleotides and the termination reaction was then incubated at 37°C for 2 minutes before addition of 4 μL of stop solution. The same primers were used for PCR and sequencing, except that for exon 4 the primer (5′-3′), CTCGTCCTTCAGGTCCACTG, was used since, for unknown reasons, poor sequence was obtained with the exon 4 PCR downstream primer.

Results

Clinical Features

The 7 patients presented with chest pain typical of angina pectoris (n=4), with exertional dyspnoea (n=1), or with sudden cardiac death (n=2) (Figure 5.1, Table 5.1). Two patients died suddenly shortly after presenting with chest pain. Electrocardiography, obtained in 6 cases, demonstrated reduction in standard lead voltages and a pseudo-infarct pattern (characterised by deep Q waves in the inferior and septal leads) in each patient (Figure 5.2). First degree atrio-ventricular block was present in one case. Echocardiography, performed in 3 cases, demonstrated massive concentric thickening of the left ventricular wall, mean approximately 18 mm, with small internal left ventricular dimensions and good systolic function; Doppler studies showed a restrictive filling defect in each case. There was no evidence of atheromatous coronary artery disease at angiography or at autopsy (3 cases each). Ventriculography showed normal contractility in each of 3 cases and mild mitral regurgitation in 2 patients. LVEDP was elevated to about 25 mmHg in each case, and pressure tracings showed a restrictive (deep-plateau) pattern. Pulmonary artery pressure was elevated to approximately 45/25 in the 3 patients studied and the cardiac index was within normal limits. Twenty-four hour ambulatory ECG monitoring was performed in 2 patients, and in each demonstrated frequent ventricular extrasystoles; ventricular tachycardia was induced during electrophysiological studies in one patient and abolished following oral amiodarone therapy, 800 mg daily, for one week.

Evidence of clinically significant amyloid deposition outside the heart was apparent at presentation in only one case (Table 5.1, patient III.5), in whom there were neuropathic features typical of FAP. Autonomic and peripheral neuropathy developed 2 years after presentation in only one other case, patient III.3. No patient had clinical evidence of nephropathy.

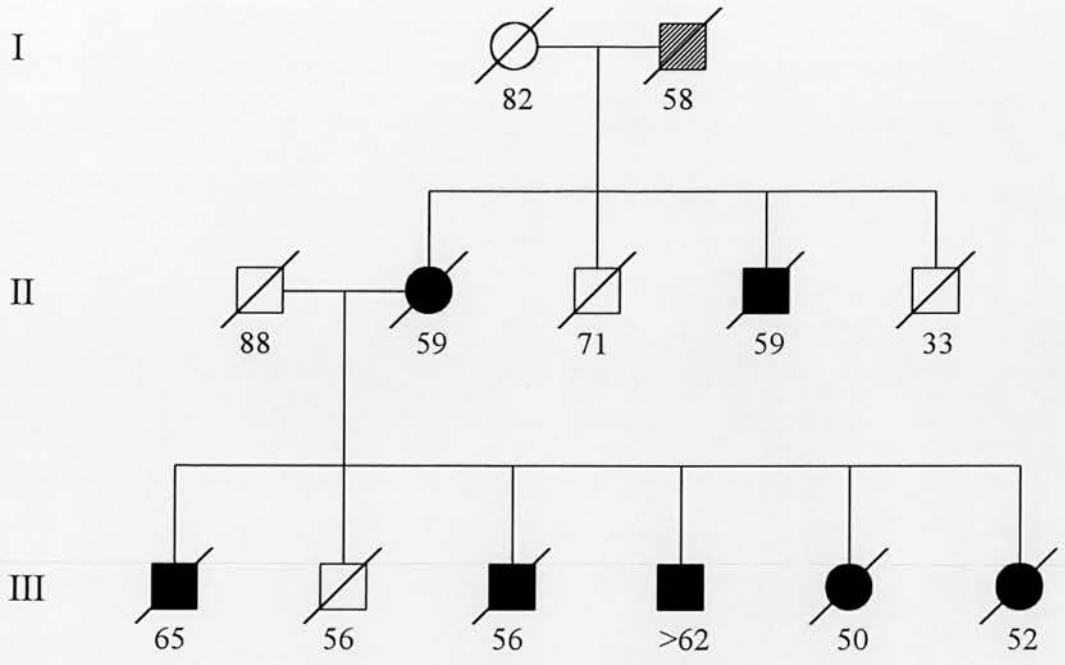

Figure 5.1 Family tree of the Italian kindred with hereditary cardiac amyloidosis. Individual I.2 (hatched symbol) is suspected of having had amyloidosis. The only clinically affected living member of the family, individual III.4, is aged 62.

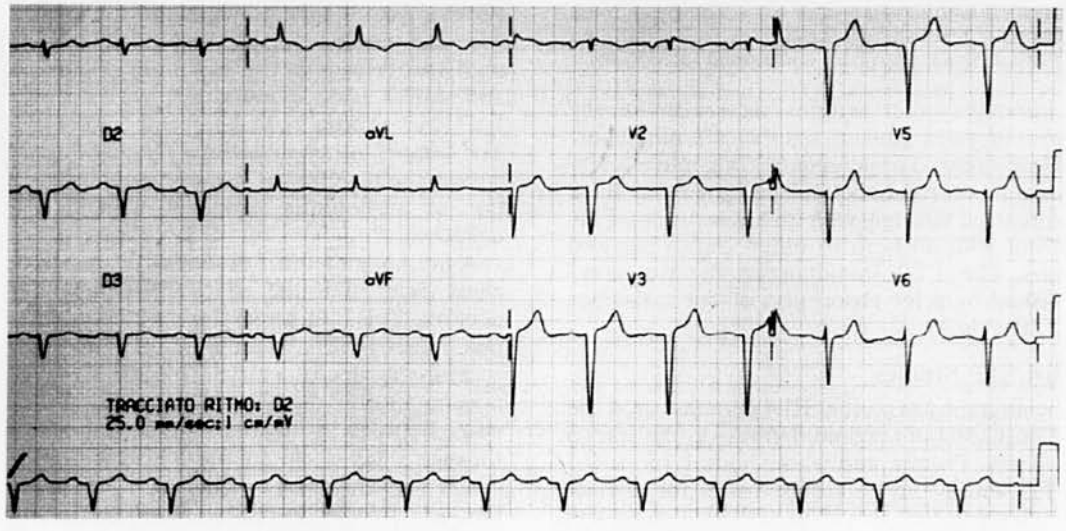

Figure 5.2 Electrocardiogram of individual III.3, showing the low voltage in the standard leads and the infero-septal pseudo infarct pattern which characterised the electrocardiograms of all cases in whom they were available.

Histology and Immunohistochemistry

Congo red stains confirmed the presence of abundant amyloid deposits in all biopsy specimens from each of the 7 cases. Cardiac histology was similar in each case and showed regularly arranged muscle fibres exhibiting hypertrophy or atrophy. There was some interstitial fibrosis and very extensive amyloid deposition. The intramyocardial arteries were also heavily infiltrated with amyloid, causing significant luminal narrowing in some areas. The abundant Congophilic amyloid deposits in myocardial and rectal biopsy tissue from patient III.5 all reacted strongly with an antiserum to TTR (Figure 5.3), but not with antisera to other known amyloid fibril proteins. The TTR immunoreactivity was completely abolished by prior absorption of the antiserum with pure TTR (Figure 5.3).

Radiolabelled SAP Studies

^{123}I-SAP scintigraphy in patient III.4 demonstrated the presence of unsuspected amyloid deposits in the spleen and both kidneys. Plasma and whole body turnover of the tracer fell within our reference range for normal subjects [60], indicating that less than 10% of the activity had localised to amyloid, consistent with a relatively modest whole body amyloid load.

Table 5.1 Clinical Details of Affected Family Members

Patient	II.2	II.4	III.1	III.3	III.4	III.5	III.6
Sex	F	M	M	M	M	F	F
Age, yrs							
Disease onset	58	59	64	53	59	49	52
Death	59	59	65	56	Alive	50	52
Cause of death	CHF	SCD	SCD	-	-	CHF	SCD
Presenting features	Dyspnoea, cardiomyopathy	SCD	Chest pain	Chest pain, dyspnoea	Chest pain	Peripheral neuropathy, gut involvement, chest pain, dyspnoea	SCD
Clinically significant extra-cardiac amyloid	None	None	None	Peripheral and autonomic neuropathy and gastro- intestinal amyloid 2 years after cardiac presentation	None	Peripheral and autonomic neuropathy, gastro- intestinal amyloid	None
Histological evidence of amyloid	Rectum	Heart at autopsy	Heart at autopsy	Heart, small intestine	Heart*	Heart, rectum, sural nerve	Heart at autopsy

*Splenic and renal amyloid deposits also demonstrated by ^{123}I-SAP scintigraphy
CHF, congestive heart failure; SCD, sudden cardiac death.

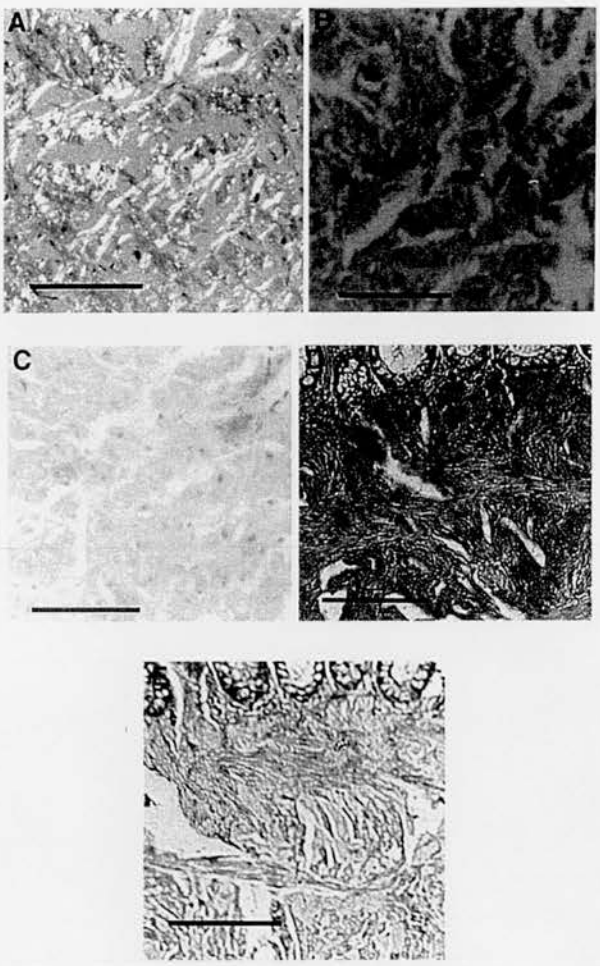

Figure 5.3 TTR amyloid in tissues of patient III.5. **A,** Myocardium stained with Congo red and viewed in crossed polarised light, showing massive infiltration with brightly birefringent amyloid deposits. **B,** Myocardium stained with monospecific antiserum to TTR, showing extensive dark peroxidase reaction product. **C,** Myocardium stained with antiserum to TTR which had been absorbed with isolated pure TTR, showing complete absence of reactivity. **D,** Rectal submucosa stained with monospecific antiserum to TTR, showing extensive dark peroxidase reaction product. **E,** Rectal submucosa stained with antiserum to TTR which had been absorbed with isolated pure TTR, showing complete absence of reactivity. All illustrations are at the same magnification; the magnification bars correspond to 100 μm.

Characterisation of the TTR Gene

Amplification and direct sequencing of all four exons of the TTR gene [145] in patients III.3 and III.4 showed that they were heterozygotes with a single base change

in one allele, altering the codon for residue 59 of the native protein from ACA (Thr) to AAA (Lys) (Figure 5.4). The remainder of the sequence was normal in both alleles.

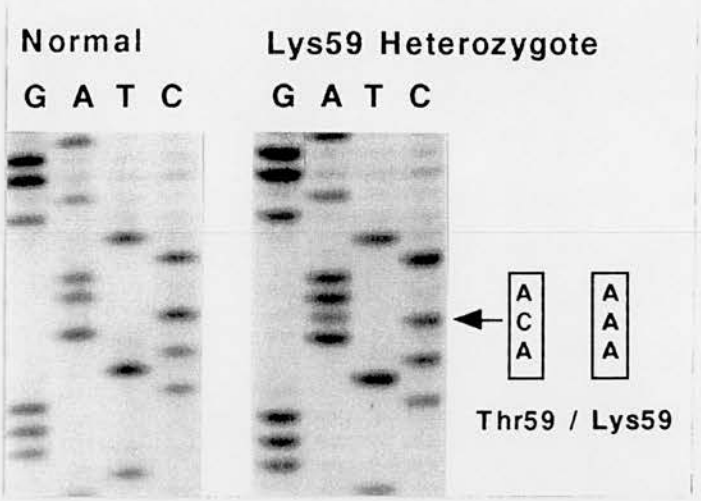

Figure 5.4 Nucleotide sequence of part of exon 3 of the TTR gene from patient III.3 (right), compared to a healthy subject (left). The patient was a heterozygote for a single base change in one allele altering the codon for residue 59 from the wild type ACA encoding Thr to AAA encoding Lys. An identical result was obtained in patient III.4, the only other family member from whom DNA was available.

Discussion

Members of two, and probably at least three, generations in this family have been affected by amyloid heart disease apparently transmitted as an autosomal dominant trait. The presence of the TTR Lys59 gene mutation, TTR in the amyloid deposits and the absence of any other TTR variant provide compelling evidence that this mutation, which has not been reported previously, is causative. Normal wild-type TTR is inherently amyloidogenic, being deposited in acquired senile amyloidosis [143], and more than 40 different point mutations in the TTR gene have been associated with hereditary amyloidosis [41, 110]. The structure of TTR has been defined to atomic resolution and each protomer consists of an α-helix, 8 β-strands forming two

face-to-face sheets, and their connecting loops [146]. Mutations in β-strand C, the CD loop, and β-strand D region are particularly likely to be amyloidogenic [147]. 59^{Thr} is situated in this part of the molecule and is highly conserved in mammalian and avian TTR sequences [148], suggesting that it is important for the molecular structure and/or function of TTR. Indeed displacement within the CD loop and D strand, which may be induced even by quite distant substitutions, has been proposed as the final common pathway for TTR fibrillogenesis via aggregation of edge β-strands [147].

Hereditary TTR amyloidosis usually presents as FAP with peripheral and autonomic neuropathy dominating the clinical picture, although the amyloid is always systemic and symptomatic involvement of the heart and kidneys is common. However, six TTR variants (45^{Thr}, 60^{Ala}, 60^{Leu}, 89^{Gln}, 111^{Met} and 122^{Ile}) have been reported in association with predominantly cardiac amyloid and minimal signs elsewhere [149]. The clinical features of affected members of the present family were very diverse (Table 5.1), with amyloid involvement of different organ systems and at ages between the fifth and the seventh decade. One subject, III.5, presented with severe neuropathic features identical to those typically seen in FAP, and similar features developed in her brother, subject III.3, 2 years after he had presented with cardiac disease. The factors, other than the TTR mutation itself, which govern the penetrance, age of onset and tissue distribution of amyloidosis associated with variant TTR are unknown.

A prominent feature among patients in this family was chest pain, with the characteristic quality of angina pectoris. This is an unusual symptom of cardiac amyloidosis, although it has been described before, and its basis is probably multi-factorial. Increased ventricular wall tension during diastole, infiltration of the interstitial space between myocardial cells and reduced blood flow through small distal coronary arterioles, the lumens of which were partially obliterated by amyloid in this family, may all contribute. Three family members had sudden cardiac deaths suggesting a dysrhythmic aetiology. Potentially life-threatening ventricular arrhythmias were detected in electrophysiological studies of 2 surviving individuals which, notably, responded to conventional therapy with amiodarone. The rhythm disturbances may have been precipitated directly by amyloid deposits and/or by ischaemia.

This family study highlights some of the common difficulties faced by the clinician when a diagnosis of cardiac amyloid is suspected. The clinical features masqueraded as those of ischaemic heart disease, and echocardiography suggested severe left ventricular hypertrophy raising the possibility of hypertrophic cardiomyopathy. Endomyocardial biopsy is essential to confirm the presence of cardiac amyloid, but unless immunohistochemical studies are performed in addition to Congo red histology, the type of amyloid cannot be defined. The clinical features of amyloid neuropathy and heart disease in patient III.5 were originally presumed to be those of AL amyloidosis which frequently presents in this manner. A family history should routinely be sought, and if more than one first degree relative has cardiac amyloidosis or if the amyloid is found to be of TTR type in a non-elderly patient, analysis of the TTR gene should be undertaken.

Restriction fragment length polymorphism and single strand conformational polymorphism analyses have been widely used to seek TTR gene mutations, and even more sophisticated indirect approaches, such as PCR-primer-introduced restriction analysis, have been reported [150]. Restriction fragment length polymorphism analysis is not applicable for the present mutation because it neither creates nor abolishes a restriction enzyme site. However, the most precise and unambiguous method for detecting mutations, with the least potential for erroneous results, is direct sequencing, as reported here [151]. This can be undertaken in any routine molecular genetic laboratory.

Characterisation of the molecular defect causing hereditary cardiac amyloidosis in our family has implications for treatment. The circulating TTR is produced almost exclusively in the liver and we have previously shown that orthotopic liver transplantation in familial amyloid polyneuropathy due to TTR mutations eliminates variant TTR from the circulation and is followed by clinical improvement [50, 116]. More than 70 cases of TTR associated FAP have now been treated by liver replacement and the majority have benefited clinically (Proceedings of the First International Workshop on Liver Transplantation in FAP, Stockholm, September 1993, unpublished). SAP scintigraphy, a quantitative method for surveying the whole body

distribution and extent of amyloid, has shown that the systemic amyloid deposits regress significantly within 1-2 years after surgery [50]. These findings are consistent with those we have obtained in systemic AA and AL amyloidosis, in which major regression of amyloid frequently also occurs when the supply of the amyloid fibril precursor protein is substantially reduced [12, 49].

Another life-saving option in our family is cardiac transplantation, although without simultaneous liver replacement cardiac amyloid deposition is likely to recur. Even if the time course of amyloid deposition in the donor heart was slow, and indeed it may take decades, it is probable that clinically important amyloid in other organ systems would develop. In July 1992 we performed simultaneous heart and liver transplantation in a 62 year old man with FAP associated with the 77^{Tyr} variant of TTR. He is alive and well with increased general well-being, having gained weight, and with subjective and objective electrophysiological evidence of improved autonomic and peripheral nerve function. This radical but potentially curative approach is currently under consideration in the present family.

Transthyretin Asp18Glu, a new variant associated with familial amyloid polyneuropathy.

Introduction

Familial amyloid polyneuropathy (FAP) is an adult onset autosomal dominant disease characterised by systemic amyloid deposition which most frequently involve the nerves, heart, vitreous and kidneys [1]. The disease is most commonly associated with variant plasma transthyretin (TTR) and more than 40 different variants have now been identified [41]. A novel TTR variant in a woman with TTR amyloidosis is described here.

Material and Methods

The proband, who was originally from Colombia, first presented age 51 years with floaters in her right eye which was unsuccessfully treated with laser therapy. The following year investigations for symptoms of heart failure revealed cardiac amyloidosis on biopsy. She then developed features of autonomic neuropathy characterised mainly by postural hypotension and diarrhoea. Although there were no symptoms referable to the peripheral nervous system, nerve conduction studies demonstrated a degree of small fibre neuropathy but no evidence for large fibre disease. Her floaters were successfully treated by excision of the vitreous opacities.

There was a strong family history of heart disease in the proband's family who remains in Colombia. Her mother died age 61 years of heart disease. Both her siblings and one maternal uncle also had heart disease. She was married but have no children.

Cardiac biopsy and the excised vitreous mass were examined by Congo red staining [4] and by immunohistochemical staining using a wide range of antibodies directed against known amyloid proteins [113]. Amyloid fibrils extracted from the vitreous mass was characterised by immunoblotting with anti-TTR antibodies [37]. DNA extracted and amplified from the proband's white blood cells was subjected to

direct dsDNA sequencing [113]. A ^{123}I-labelled SAP scan, an *in vivo* technique for the identification of systemic amyloid deposits was performed [58, 59].

Results

Amyloid deposits in the cardiac biopsy and vitreous mass were identified by Congo red staining. Immunohistochemical studies of vitreous amyloid demonstrated positive staining only with anti-TTR antibodies and the staining was abolished by absorption with pure human TTR. Immunoblot of fibril proteins extracted from the vitreous mass confirmed that the fibrils are derived from TTR. Direct dsDNA sequencing confirmed that the proband was heterozygote for a novel point mutation in exon two of the TTR gene resulting in a codon change from GAT to GAG, encoding a single amino acid substitution of aspartic acid by glutamic acid in position 18 of the mature protein (Figure 5.5). There was no evidence for significant extra-cardiac systemic amyloid deposits on scintigraphy.

Discussion

Genetic analysis revealed a new TTR variant, Glu18, in a patient with TTR amyloidosis. The genotype-phenotype relationship between TTR variant and TTR amyloidosis is well established [1, 14, 41] and this patient's clinical course is consistent with disease due to FAP. The hereditary nature of the disease is suggested by the strong family history of heart disease but unfortunately tissue was not available for analysis to demonstrate the Mendelian inheritance in this kindred.

The TTR subunit is rich in β-sheet structure [152], a property commonly found in amyloid precursor proteins and indeed may be an important factor determining the amyloidogenecity of wild-type TTR associated with senile systemic amyloidosis. Point mutations encoding single substitutions appear not only to increase the amyloidogenic potential of the TTR molecule, probably by a destabilising effect on the secondary structure of the protein, but also results in disease of a different phenotype. However,

not all TTR mutations are amyloidogenic [41] and amongst the 40 amyloidogenic variants, there is no obvious pattern to the substitutions which are widely distributed throughout the length of the protein, although there are several mutation hot spots. There is also considerable heterogeneity in the clinical spectrum of FAP, even amongst kindreds with the same mutation, suggesting that factors other than the mutations are important in determining pathogenesis in this apparently monogenic disease. The importance of these non-genetic factors is further illustrated by the finding that unlike most autosomal dominant diseases, gene dosage may not adversely influence the age of onset and severity of symptoms in FAP. Indeed there are individuals homozygous for TTR Met30 who have remained asymptomatic throughout life [153].

Our description of TTR Glu18 adds to the increasingly heterogeneous mixture of TTR variants associated with FAP. This is also the first Colombian family reported to have the disease which most commonly affect populations in Portugal, Sweden, and Japan. However, with the increasing recognition of this disease and ready availability of routine molecular biological investigative techniques, the wider population distribution of the disease will continue to be increasingly recognised and reported.

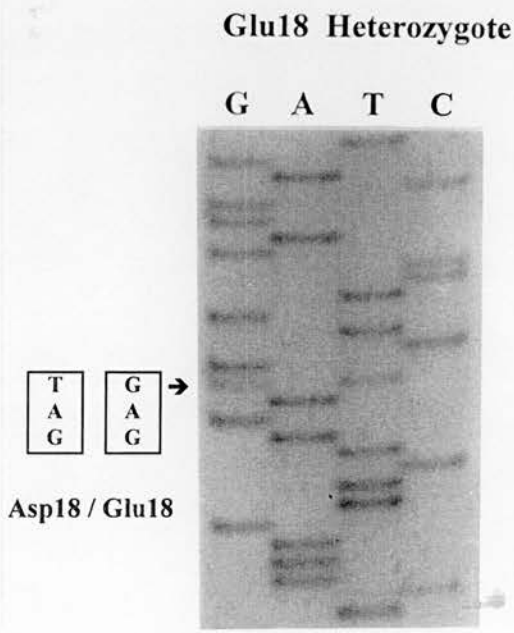

Figure 5.5 Nucleotide sequence of part of exon 2 of TTR gene of the patient, showing that she was heterozygous for a point mutation resulting in codon change from GAT to GAG which encodes substitution of wild type residue (aspartic acid) by glutamic acid at position 18 of the mature protein.

Chapter 6 - Dialysis-related amyloidosis

Long term effect of renal transplantation on dialysis-related amyloid deposits and disease symptomatology: A prospective 5 year study

Introduction

Dialysis-related amyloid (DRA), a potentially crippling complication of dialysis-dependent renal failure, is now a major cause of morbidity in long-term dialysis populations [1, 65]. Its major clinical manifestations are related to the accumulation of amyloid deposits within synovial membranes, resulting in carpal tunnel syndrome (CTS) and dialysis arthropathy which is characterised by arthralgias of the large and medium sized joints. Although articular tissue is the main site of deposition, systemic distribution of DRA is a well described and common but late event that may become clinically significant [154]. The precursor protein of DRA deposits is β2-M [23], a low molecular weight protein expressed by all nucleated cells which is normally catabolised by renal tubules. However, this tubular function of normal kidneys is poorly replaced by dialysis, causing uraemic retention of β2-M, a process that is evidently necessary for the pathogenesis of the disease [1, 65].

Specific treatment of DRA is therefore aimed at increasing clearance of β2-M which is not removed by conventional haemodialysis (HD) with standard cuprophane membranes. Continuous ambulatory peritoneal dialysis (CAPD) [155], high flux dialysis [156], immunoadsorption columns [157], and especially haemofiltration [158] may all reduce but not normalise β2-M levels, a metabolic event that can only be achieved effectively with a successful renal transplant (T_x). In a recent radiological study [159], restoration of normal β2-M metabolism following a successful renal T_x was accompanied by rapid resolution of the clinical features of DRA, and a halt in disease progression.

We have recently [160] described a scintigraphic method for the non-invasive *in vivo* diagnosis of DRA which is based on the specific binding of serum amyloid P (SAP) component, a universal constituent of all amyloid deposits [35], to amyloid fibrils. In that study [160], we demonstrated that ^{123}I-labelled SAP is sensitive and specific for the diagnosis of DRA in long-term HD patients. We now report results of a prospective follow-up study on this original cohort of patients to assess the effect of renal transplantation on clinical, radiological, and scintigraphic features of DRA deposits, compared to a control group of patients who remained on HD.

Methods

Patients

Fifteen patients with clinical and histological evidence of DRA, (aged 41-66 years, 10 males, 5 females) who had undergone haemodialysis for a median of 18 years (interquartile range, 14-21 years) were studied prospectively for 5 years. At completion, 6 patients (Table 6.1) were still receiving dialysis (HD group) whereas 9 (Table 6.2) had successfully undergone renal transplantation (T_x group). Three T_x patients had been transplanted shortly before recruitment. The median duration post transplantation was 4.5 years (range, 2-7.5 years). The HD and T_x groups were similar with respect to follow-up period, age, sex and total duration of dialysis.

Table 6.1 Clinical details of haemodialysis patients; data on entry into the study in ( )

Patient	Sex	Age, years	Dialysis duration, years	Scan interval, years	β2-M, mg/L	Creatinine, μmol/L	Number of CTD
LG	M	63.6	15.7	5.6	29.5	481	3(3)
BJ	M	52.2	21.3	5	29.5	867	3(0)
IM	F	63.7	13.8	3.8	29.8	773	2(2)
EM	M	63	20.3	5.3	38	1064	3(2)
PP	F	66.7	8.2	2.3		434	0(0)
SO	M	53.8	15.9	3.2	56	1443	0(0)
Median {IQR}		63.3 {56.1-63.7}	15.8 {14.3-19.2}	4.4 {3.4-5.2}	29.8 {29.5-38}	820 {554-1015}	2.5{0.5-3} (1{0-2})

IQR, Interquartile range; CTD, carpal tunnel decompression

Table 6.2 Clinical details of renal T_x patients; data on entry into the study in ( )

Patient	Sex	Age, years	Dialysis duration, years	Scan interval, years	Post-T_x, years	β2-M, mg/L	SAP, mg/L	Creatinine, μmol/L	Number of CTD
PR	M	50.8	21.4	5.2	5.9	3.2	30	139(1360)	2(2)
BS	M	65.3	13.3	5.1	7.5	2.6	22	70(1150)	1(1)
BT	M	47.8	21	5	6.3	8.3	23	391(1230)	2(2)
AS	F	51.3	18.3	4.4	2.8	2.6(39)	19	80(501)	2(2)
ES	M	63.7	19.9	4.6	2	2.4(46)	23(39)	105(951)	1(1)
DW	M	55.9	18.8	4.8	4.2	7.7(49)	48(47)	207(970)	0(0)
EW	F	63.2	14.3	4.6	3	3.3(45.5).	20(40)	123(630)	0(0)
CF	M	41.2	17.8	4.9	4.4	3.4(45.5)	31(72)	117(728)	0(0)
ShO	F	51.1	21	5.2	4.9	2(38)	24(12)	82(954)	4(4)
Median {IQR}		51.3 {50.8-63.2}	18.2 {17.8-21}	4.9 {4.6-5.1}	4.4 {3-5.9}	3.2{2.6-3.9} (45.5{40.6-45.9})	23{22-30} (40{39-47})	117{82-139} (954{728-1150})	1{0-2} (1{0-2})

IQR, interquartile range; CTD, carpal tunnel decompression

Assessment

Symptoms

Clinical features of dialysis arthropathy were evaluated by a rheumatologist. At baseline each affected hip, knee, shoulder and wrist, including carpal tunnel syndrome (CTS), scored one point (maximum 8 points). At completion, each joint scored 2 if worse (maximum 16 points), one if unchanged, 0.5 if improved, or zero if features had resolved. An additional point was given for any joint affected at follow-up which had been normal at baseline. The relationship between symptoms, renal transplantation and corticosteroid dosage was recorded.

Radiology

X-rays of the hands, wrists, knees, shoulders and hips were obtained at approximately yearly intervals. Previous films were available in each case. The baseline radiologic joint score comprised one point for each amyloid bone cyst identified using rigorous criteria as summarised in Table 6.3 [161]. At completion, cysts that were larger scored 2, one if unchanged and 0.5 if smaller. No cyst resolved completely and new cysts scored an additional point. Radiographs were evaluated "blind" by an experienced skeletal radiologist. In two cases some X-rays were missing.

^{123}I-labelled SAP scans and metabolic studies

Isolation and radiolabelling of human SAP with ^{123}I and ^{125}I, scintigraphy, and turnover studies were performed as described [58-60], although the latter were necessarily limited to plasma clearance measurements in anuric patients. Scintigraphs were assessed anonymously by two physicians with experience of 1000 such studies. Serial scans were unavailable for the wrists in one patient and knees in another. Baseline images of the shoulders, wrists, knees and hips were scored one point for each joint in which there was abnormal uptake of tracer (maximum 8 points). At completion each joint scored 2 points if uptake was greater (maximum

16 points), one if unchanged, 0.5 if improved, or zero if the images fell within normal limits; an additional point was given for any joint that became abnormal during the study.

Table 6.3 Radiological characteristics of DRA bone cysts *

At least 5 mm diameter in the wrists At least 10 mm diameter in the shoulders and hips
Multiple
Affect at least 2 joints
Periarticular in distribution
Joint space adjacent to cysts must be normal (to exclude subchondral bone cysts of osteoarthritic origin)
Grow in size by > 30% per year (to be considered significant)
Located outside areas prone to have "synovial inclusions" (such as upper third of femoral neck or anatomical humeral neck)
Located outside weight bearing area of the joint (such as outer third of acetabulum)

*Kidney International 1991;Vol 39:1012-1019

Histology

Biopsies obtained during the study were subjected to routine histologic examination, staining with Congo red [4] and immunohistochemistry with a panel of antisera against amyloid fibril proteins [162].

Serum concentration of β2-M, SAP and creatinine

β2-M and creatinine levels were measured in venous blood using standard autoanalyser techniques. SAP concentration was determined by electroimmunoassay [163].

Statistical analysis

Demographic differences between the patient groups and results of metabolic measurements were sought using the Mann-Whitney U test. Scores for symptoms, scans and X-rays were analysed using the Wilcoxon rank tests for paired samples and unpaired groups. $P < 0.05$ was deemed significant.

Results

Symptoms

At baseline all patients had symptoms of DRA, with the same median of 4 affected joints in each group. Surgery for CTS had been performed a median of once per patient in each group.

Four of the 6 HD patients experienced worsening arthralgia, and recurrence or development of CTS (Table 6.1), 2 of them requiring surgery. The two patients without progressive symptoms had the shortest follow up.

In the T_x group, 8 of 9 patients reported rapid and substantial relief of arthralgia following transplantation ($P = 0.01$ for scores at entry and end of study) (Figure 6.2). This was sustained despite reduction ($N = 5$) or complete withdrawal ($N = 4$) of corticosteroids, although symptoms recurred transiently in 2 cases whilst tapering. No T_x patient required surgery for CTS. DRA symptoms remained after transplantation in one case.

The difference between symptom scores for the HD and T_x groups at completion was significant, $P = 0.018$. The difference between the changes in scores from entry to end of study in the two groups was also significant ($P = 0.018$).

Radiology

Amyloid bone cysts were most common in the wrists followed by the hips, shoulders and knees (Table 6.4). Among patients with baseline radiologic abnormalities, the median number of affected joints per patient was 3.5 in the HD group and 3 in the T_x group, whilst the median number of cysts per affected joint were 2 and one respectively.

Among the HD patients, 18 DRA bone cysts in four cases were identified at baseline. At follow-up (Table 6.4, Figure 6.3) the cysts were larger and there were 7 new cysts, a significant deterioration ($P = 0.018$). In contrast, among the 5 T_x patients with baseline radiographic abnormalities, cyst size decreased and there was ossification in the cyst walls (Figure 6.7) in 4 affected joints in 4 patients. None of the 24 cysts resolved (Table 6.4, Figure 6.4), but there was a significant difference between the HD and T_x scores at completion ($P = 0.02$). Changes in score during the study were also significantly different between the groups ($P = 0.018$).

^{123}I-labelled SAP scintigraphy

Positive scans of at least the wrists were obtained at baseline in all subjects except one HD patient (Figure 6.5). Amyloid was identified frequently in the knees and, less often, the shoulders. Images of hips were non-diagnostic because of their deep location and proximity to the strong masking signal from tracer in the central blood pool. Amyloid was not identified in any other site.

DRA was progressive in 5 of 6 HD patients (Figure 6.5): 5 additional joints gave positive images and there was increased tracer uptake in 7 joints that were positive at baseline (Figure 6.8). Follow-up studies were unchanged in one HD and one T_x patient. In each of the 8 other T_x patients, amyloid deposits regressed (Figure 6.9) in at least one site (Figure 6.6) with tracer uptake reduced in 16 of the 41 joints identified as abnormal at baseline, $P = 0.01$. Amyloid decreased below limits of detection (Figure 6.10) in 6 joints in 3 patients, including all 3 affected joints in one case. Changes in joint score at baseline and completion were significantly different between the two groups, $P = 0.018$.

Histology

During the study tissue was resected at carpal tunnel release in 2 HD patients, and at spinal surgery in 2 T_x patients, one with a fractured cervical vertebra, the other during a decompressive procedure. All tissues contained amyloid that stained specifically for β2–M.

Serum concentration of β2–M, SAP and creatinine

Among T_x recipients serum levels of β2–M and creatinine were significantly lower at completion (median values 3.2 mg/litre and 117 μmol/litre respectively) than at baseline (45.5 mg/litre and 954 μmol/litre) ($P < 0.001$), corresponding with restored renal function. The concentration of SAP, which is raised in renal failure [164], also fell following T_x, but not significantly. No changes in serum levels of β2–M, SAP or creatinine occurred in HD patients.

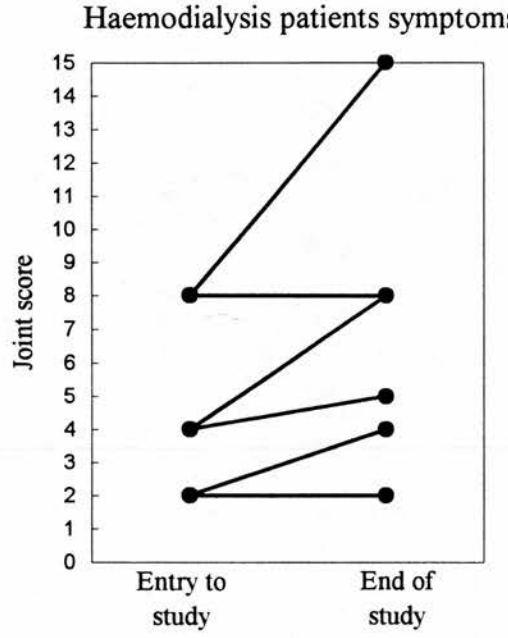

Figure 6.1 Joint scores for symptoms for individual haemodialysis (N = 6) patients at entry and end of study

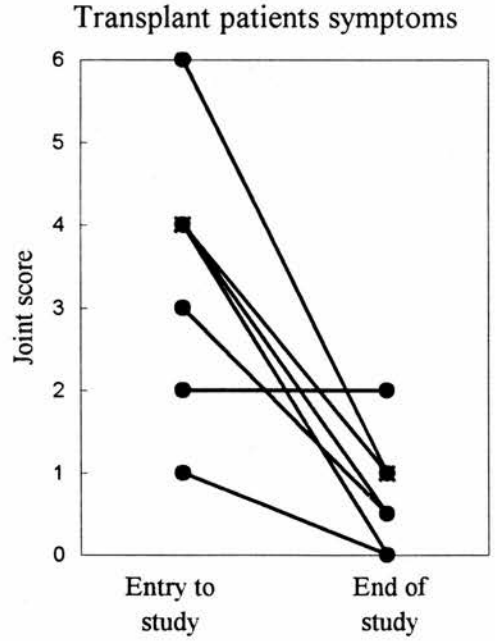

Figure 6.2 Joint scores for symptoms for individual renal transplant (N = 9) patients at entry and end of study

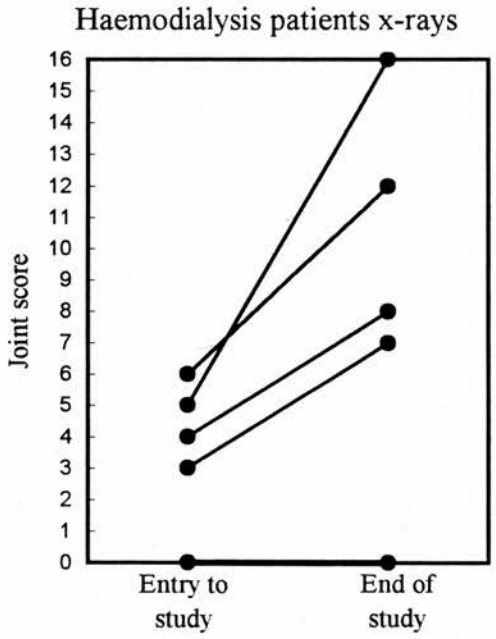

Figure 6.3 Joint scores for X-rays for individual haemodialysis patients (N = 6) at entry and end of study

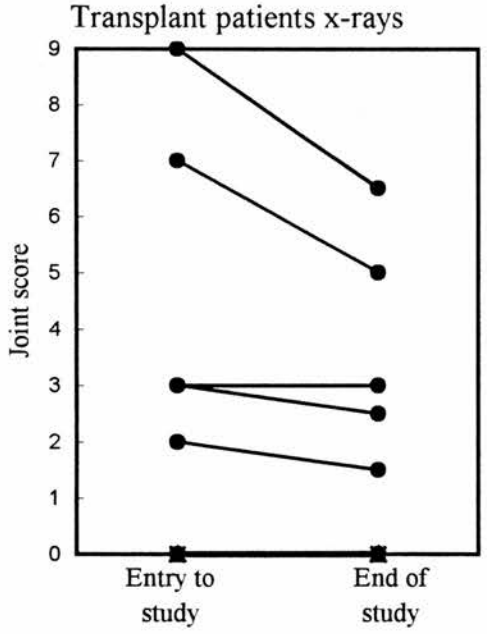

Figure 6.4 Joint scores for X-rays for individual renal transplant (N = 9) patients at entry and end of study

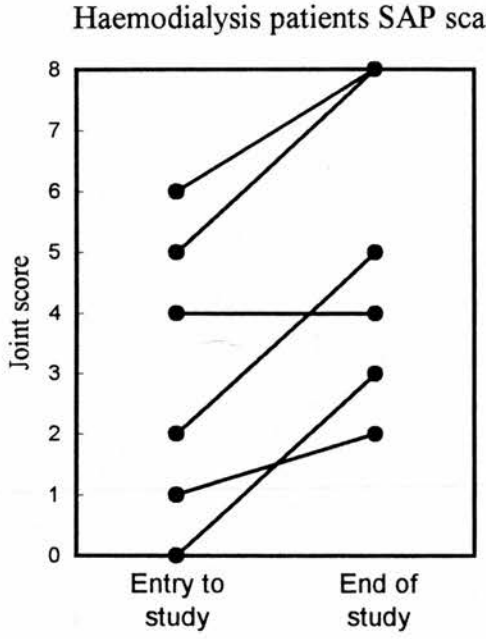

Figure 6.5 Joint scores for SAP scintigraphy for individual haemodialysis (N = 6) patients at entry and end of study

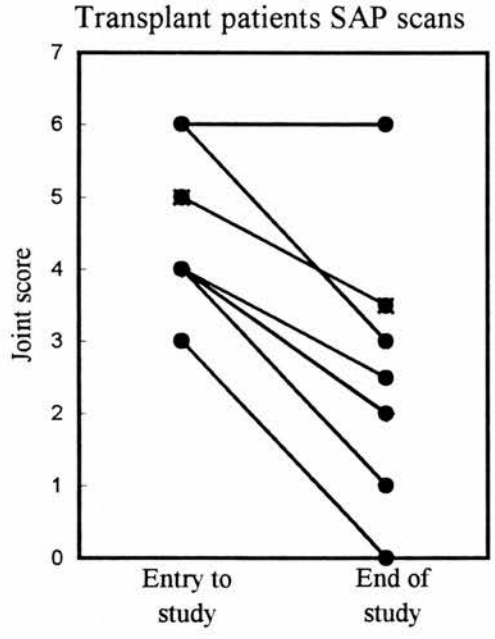

Figure 6.6 Joint scores for SAP scintigraphy for individual renal transplant (N = 9) patients at entry and end of study

Table 6.4 Number of amyloid bone cysts

	Renal transplant patients (N = 5)		Haemodialysis patients (N = 4)	
	Entry	Completion	Entry	Completion
Wrists	18	18	14	14
Shoulders	0	0	2	3
Hips	5	5	2	8
Knees	1	1	0	0
Total	24	24	18	25

Metabolic studies

Plasma clearance of ^{123}I-SAP, which reflects whole body amyloid load, was obtained in 4 HD and 8 T_x patients. The plasma concentration of ^{123}I-SAP after 6 hours, expressed as the mean (SD) percentage of the injected dose, was 75% (8) at baseline in the HD group and 69% (1) at completion, compared with 75% (4.5) and 76% (7) respectively in the T_x group. These trends were not statistically significant. Whole body retention of radioactivity fell within the normal range [60] in all T_x patients at completion.

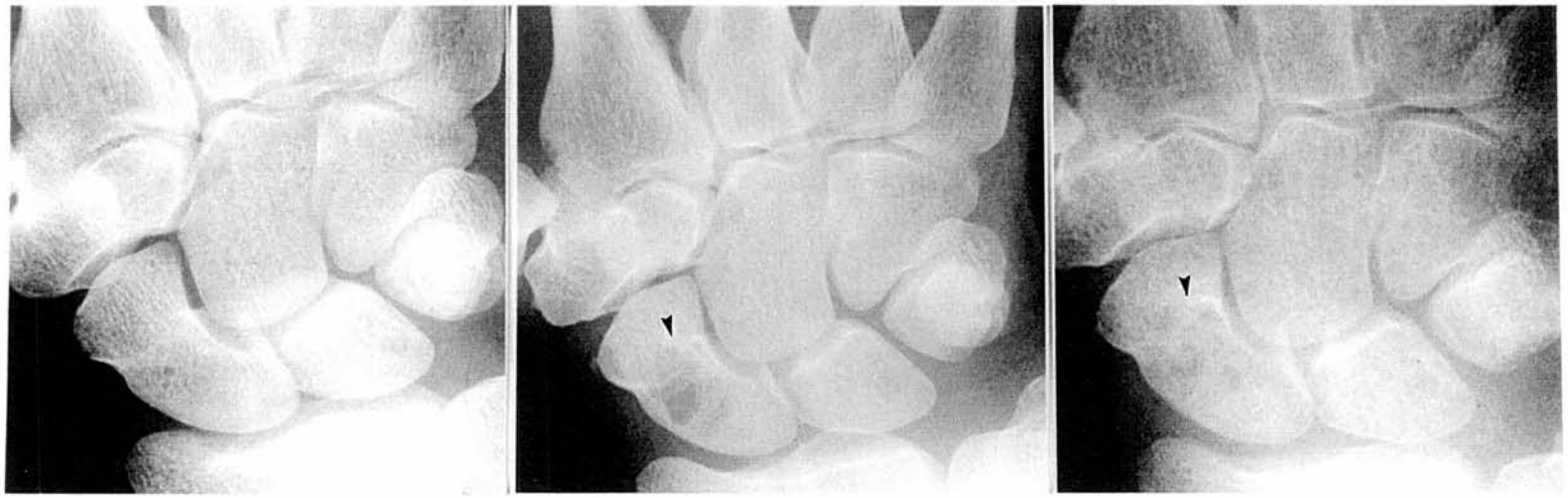

Figure 6.7 Serial wrist X-rays of a 51 year old woman, treated with HD for 18 years before undergoing successful renal transplantation in 1990. *DRA-related bone cysts (arrow), which developed in the right scaphoid between 1985 (left) and 1990 (middle), became considerably smaller within 3 years of grafting (right, 1993)*

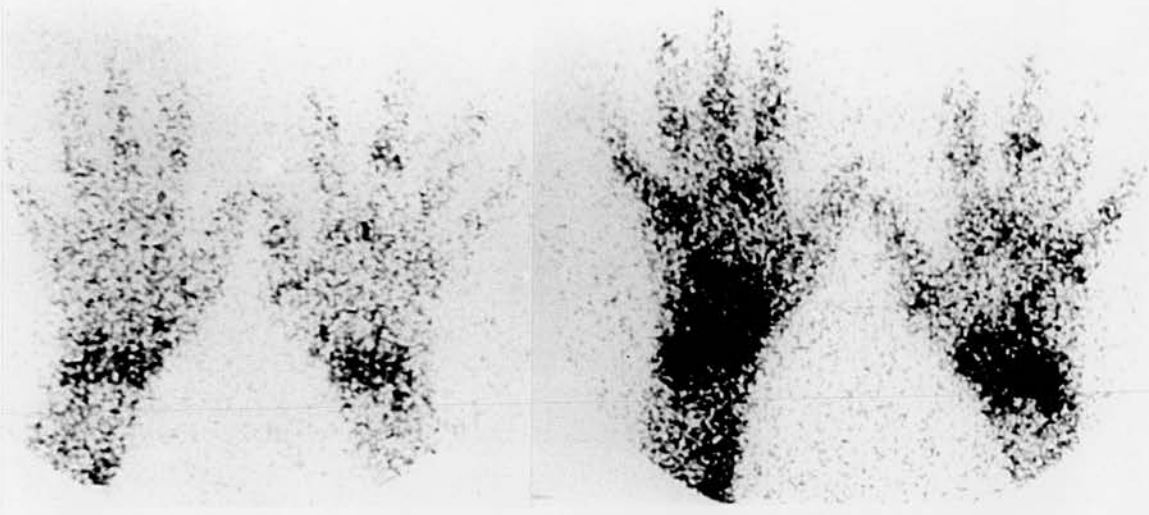

Figure 6.8 Serial ^{123}I-SAP scans showing progression of DRA in the hands of a 67 year old lady who developed symptomatic DRA in both wrists after 6 years on HD. *Images obtained in 1991 (left) show abnormal uptake of tracer into amyloid deposits in the wrists and some small joints of the hands; uptake was greater in 1994 (right).*

Correlation between clinical features, radiology, scintigraphy and histology

The most frequent presenting feature of DRA was CTS followed by shoulder arthralgia. Scintigraphy and X-rays were positive most often in the wrists. Positive scintigraphy of at least one joint was seen initially in 14/15 (93%) of patients whereas radiologic bone cysts occurred in only 9 (60%) of cases. This is much higher than the 9% of 221 patients found to have positive bone radiology in another study [161] which adopted a similarly rigorous criteria for radiological definition of amyloid bone disease. However, in that study, half the patients were dialysed on high flux membranes which were found to delay the onset of bone amyloidosis, and, in addition, radiologically affected patients had been dialysed for a mean period of only 9 years compared to the mean dialysis period of 17.4 years for our radiologically affected patients.

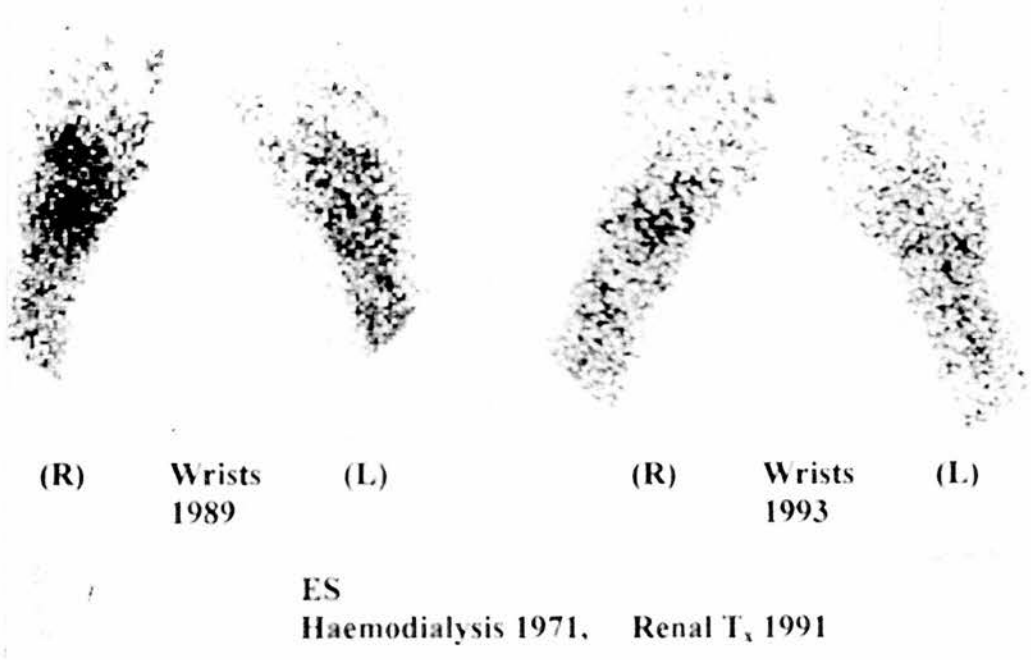

Figure 6.9 Serial ^{123}I-SAP scans showing regression of DRA in a 63 year old man, who was treated with HD for 18 years before undergoing renal transplantation in 1991. *Intense abnormal localisation of tracer to the wrists in 1989 (left), greatest in the most symptomatic right hand, is substantially reduced in the follow-up images obtained in 1993, nearly 3 years after transplantation (right).*

At completion, among 31 joints affected clinically in HD patients, DRA was evident radiologically in 11 (35%), and scintigraphically in 17 (55%). Characteristic symptoms were the sole clinical feature of DRA in 8 (26%) of the joints. Conversely, among 32 joints with radiological and/or scintigraphic evidence of amyloid, 9 (28%) were asymptomatic; 2 out of 8 joints with sub-clinical DRA at baseline became symptomatic subsequently.

All but one T_x patients experienced profound reduction of DRA symptoms whilst one or both imaging modalities provided evidence of amyloid regression in every case. Amyloid could not be detected in scans of 8/41 (20%) joints that had previously been abnormal, although this does not necessarily indicate complete resolution. Scans showed improvement in a further 16/41 (39%) joints. Radiology

also showed some improvement, but to lesser extent and among fewer joints (4/13, 31%). Overall, radiology and scintigraphy improved much less than clinical symptoms.

Baseline wrist radiology and scintigraphy had been positive in both HD patients who developed CTS requiring surgery, but spinal imaging of the 2 T_x patients who underwent surgery was normal.

Figure 6.10 Serial ^{123}I-SAP scans showing regression of DRA in a 63 year old man, who was treated with HD for 18 years before undergoing renal transplantation in 1991. *There is abnormal uptake of tracer into both knees in 1989 (left) followed by asymmetric improvement, with normalisation of the right knee signal by 1993 (right).*

Discussion

In patients with DRA, a successful renal T_x results in rapid resolution of symptoms of dialysis arthropathy [65, 159, 165]. In addition to confirming these findings, we have in our study also demonstrated that this early symptomatic relief is preserved up to 7.5 years post-T_x despite subsequent withdrawal or reduction in steroid dosage. Until recently, symptomatic relief achieved following a successful T_x was thought to be due largely to the anti-inflammatory effect of steroids [65, 159]. This was supported by a study [166] reporting the beneficial effect of low dose oral steroids in dialysis patients with DA and the observations [167] that similar improvement was seen in 2 patients primed with intravenous steroids but not grafted. However, although steroids may modulate DRA symptomatology in the short-term, our unique observations suggest that other factors operational immediately post-T_x and preserved in the longer-term with good graft function are responsible.

Amyloid fibrils was previously thought to be largely inert, causing organ damage mainly by its physical presence. However, recent *in vitro* studies have shown that amyloid fibrils may be directly toxic to surrounding cells [99, 168]. This toxic effect is seen only in cells in direct physical contact with the fibrils [99] and is mediated by the induction of apoptosis or programmed cell death. It is therefore possible that target organ damage and local disease symptomatology are caused by similar mechanisms or toxic factors induced *in vivo* by active fibril formation and deposition, a process that is halted once further fibril formation ceases following a successful T_x.

It is also possible that DRA symptomatology is dependent on the uraemic state seen in dialysis-dependent renal failure which contain uraemic toxins or "middle molecules" which are too large to be dialysed and thought to be responsible for much of the morbidity seen in patients with renal failure [169]. There are several possible candidates, including β2-M itself, which is biologically active as a bone-derived growth factor capable of inducing release of fibroblast collagenase [170] and bone remodelling [171], and these, together with its high affinity for collagen [172] are properties which may encourage active amyloid deposition in articular

tissue, a process that may as discussed, be an important local determinant of disease symptomatology.

Finally, the process of dialysis itself may play an important role in both disease pathogenesis and symptomatology [65, 165]. Development of DRA may be delayed by predominant use of high flux dialysis [161, 173, 174] and in patients who are already affected by DRA, conversion to high flux dialysis may be accompanied by substantial improvement in symptoms of dialysis arthropathy [175, 176]. Our own preliminary observations with CAPD patients [177] also suggest that clinical expression of DRA may be different with this mode of dialysis. In the present study, the most striking evidence was provided by a patient (BJ) whose symptoms of DA (which had resolved completely 2 years earlier following a successful T_x), returned rapidly within a month of recommencing HD although he was still on a small dose of steroids at that time.

Radiologically and scintigraphically, disease progression was halted in all cases and in most joints there was evidence for disease regression although this was not as striking as the clinical improvement seen post-T_x. In the only other radiological study [159] to have assessed the effect of renal transplantation on amyloid bone cysts, there was no change in the bone cysts at follow-up. We have, however, been able to demonstrate healing with either ossification or reduction in the size of bone cysts in 5 joints of 4 affected patients. There are several possible reasons for this difference in our findings. Our patients had less severe disease with 25 cysts in 5 patients and secondly, they had a longer mean follow-up period of 5.7 years post-T_x compared to the earlier study with 34 cysts in 5 patients and mean post-T_x follow-up of 3.9 years. This suggest that mobilisation of amyloid, which may vary considerably between different patients and different anatomical sites [165], may be delayed and not obvious radiologically during short-term follow-up. It is also possible that the absence of changes post-T_x could be due to failure of bone to heal because of continued treatment with critical doses of steroids which in our cohort of patients had either been reduced substantially or stopped completely. The capacity of bone to heal and reabsorb cysts is also evident in other diseases [178] and in fact regression and even complete resolution of bone cysts due to multiple myeloma has been described following successful treatment of the

underlying disease [179]. Finally, age, sex, and menopausal status of female patients may also affect the ability of bone to heal although numbers of patients are too small for any definitive conclusions to be made.

Scintigraphically, there was complete resolution of the disease in only 1 patient (EW) who was also the least severely affected clinically, radiologically and scintigraphically. The 2 other patients (BS and BT) with complete resolution of the disease scintigraphically in some joints had both been transplanted for the longest period of time. These findings support our radiological observations that once precursor β2-M levels have been normalised by a successful T_x, disease severity and duration post-T_x are two important determinants of disease regression. Site of amyloid deposition may also be an important determinant. The absence of complete resolution of bone cysts radiologically together with the delay in improvement in radiological signs suggest that amyloid present in bone cysts cannot be mobilised as rapidly as amyloid in other articular structures such as the synovium or that bone cysts, present non-specifically in up to 30% of non-uraemic patients [180], and in uraemic patients may also be due to secondary hyperparathyroidism [181], are a relatively crude measure of the spectrum of DRA.

Histologically, tissue was available for examination from only 1 patient (BT) post-T_x when he fell and fractured his cervical spine which had to be stabilised surgically by fusion of C5 and C6 vertebrae. Bone biopsy obtained at operation was positive for amyloid although there was no radiological evidence for amyloid bone disease in the cervical spine or other joints. This case illustrates very clearly that negative radiology does not exclude DRA and equally importantly, it also demonstrates that in patients with DRA, despite disease regression post-T_x, complete reabsorption of amyloid and bone healing may be delayed considerably and may therefore continue to cause problems such as pathological fractures many years after a successful T_x.

Amongst HD patients, several asymptomatic joints that had scintigraphic evidence for DRA became clinically affected at follow-up, confirming previous observations [160] that SAP scintigraphy may detect subclinical or pre-symptomatic lesions that are well recognised in DRA [182]. In fact, in a recent post-mortem study [183], histological prevalence of DRA was found to be higher than suggested

clinically, frequently preceding clinical features of the disease. An unexpected finding was the common involvement of the knees, which was affected histologically more frequently than the shoulders, and least frequently in the hips, contrary to what might be expected from clinical features of the disease. This interesting finding however, is consistent with our both our present and previous scintigraphic studies of patients with DRA, where we found that knees were affected more frequently than the shoulders. In addition to supporting our scintigraphic findings, these histological observations also suggest that clinical expression of DRA deposits are delayed and may be obvious only when it reaches a critical size or may be influenced by factors such as dialysis modality, whilst it is also the first feature of the disease to improve following a successful T_x.

In both groups of patients, CTS was the earliest and commonest clinical feature of DRA, and although all patients reported symptoms related to one or both wrists, the need for CTD, often used as the most definitive marker of CTS [161, 174] was required in only 10 of 15 patients. In contrast, positive scintigraphy was obtained in one or both wrists in all patients whilst amyloid bone cysts were seen in the wrists of 8 out of 14 patients from whom serial radiology was available. These findings suggest that SAP scintigraphy is the most sensitive non-invasive marker in detecting both symptomatic and pre-symptomatic DRA deposits, and the prevalence of the disease is frequently underestimated clinically and radiologically.

The rigorous radiological criteria that we used for X-ray confirmation of DRA is specific for amyloid bone cysts but lacks sensitivity which can be improved if interpreted in conjunction with clinical and scintigraphic findings. The need to demonstrate bone cysts in at least 2 joints as one of the several criteria for confirmation of the disease obliged us to classify as radiologically "normal", 1 patient (ES) who had a larger than 10 mm cyst in his L shoulder and another patient (PP) who had several larger than 5 mm cysts in her L wrist, although in both cases there was clinical and scintigraphic evidence for the disease. Appropriate modification of this strict radiological criteria may therefore improve sensitivity without necessarily affecting specificity of the radiological diagnosis for this disease as have been employed by some specialist units [174] which accept bone cysts of appropriate size and location in only 1 joint to be consistent with DRA.

Finally, symptomatic treatment of DRA with steroids and non-steroidal anti-inflammatory drugs are likely to have only a limited short-term benefit in the face of the progressive nature of the disease as are surgical procedures such as CTD which often have to be repeated. Immunoadsorption columns and dialysis modalities such as haemofiltration, high flux dialysis and use of ultrapure dialysis fluid may at best delay clinical development of DRA [13]. Renal transplantation is therefore the only effective therapy for DRA, the clinical benefits of which are sustained in long-term follow-up and accompanied by both scintigraphic and radiological regression of the disease which may be delayed. These findings add to our observations in other types of amyloidosis [12, 47-50, 184, 185] that amyloid deposits are constantly being turned over, and if the supply of precursor proteins is substantially reduced, the halt in further amyloid deposition or disease progression is frequently accompanied by disease regression. In addition to patients with AA [12, 49], AL [47, 48, 185], and hereditary transthyretin amyloidosis [50], this has now been demonstrated in serial clinical, radiological and scintigraphic studies of patients with DRA or β2-M amyloidosis.

Dialysis-related amyloid and treatment with continuous ambulatory peritoneal dialysis: A clinical radiological and scintigraphic study

Introduction

Although dialysis-related amyloid (DRA) may complicate patients on continuous ambulatory peritoneal dialysis (CAPD) [1, 186-188], the perfect biocompatibility of the peritoneal membrane, its permeability to β2-M and, the lower levels of β2-M in these patients [189, 190] suggest that development of the disease may be delayed in CAPD compared to patients on haemodialysis (HD). However, comparison of the prevalence of DRA in these two patient populations is hampered by the small number of patients who have been dialysed long-term and exclusively by CAPD, and by the lack of a sensitive *in vivo* technique for the diagnosis of DRA. We have previously described the use of ^{123}I-labelled serum amyloid P component (SAP) scintigraphy as an *in vivo* technique for the diagnosis of systemic amyloidosis [58-60], including DRA [160], and have recently demonstrated that this scintigraphic technique may also be used for following the fate of amyloid deposits in patients who remain on HD compared to successfully transplanted patients [191]. We now report a clinical, radiological and scintigraphic study of DRA in 13 patients who have been dialysed predominantly by CAPD.

Patients and Methods

Thirteen patients from 5 renal units who have been on continuous dialysis for at least 5.5 years and spent at least 80% of their total dialysis time on CAPD were studied (Table 6.5). None of these patients had evidence for systemic diseases as a cause of their renal failure. Patients were evaluated clinically for features of carpal tunnel syndrome and for arthralgias of the large and medium sized joints characteristically seen in dialysis arthropathy [65]. Annual X-rays taken of the wrists, shoulders, hips and knees in all patients were read 'blind' by a skilled skeletal radiologist for evidence of amyloid bone cysts using a criteria as previously described (Table 6.3) [161]. *In vivo* assessment of amyloid deposits was performed

scintigraphically with radio-labelled SAP. Images were taken 24 hours after injection of tracer and all metabolic studies were performed as previously described [58-60]. Scans were read 'blind' by a specialist with experience with over 800 SAP scans. Blood was also taken from all patients prior to injection of radio-labelled SAP for determination of serum β2-M levels which were measured by standard autoanalyser techniques. All values are expressed as medians with interquartile ranges and data was compared statistically using the non-parametric Mann Whitney U test.

Results

Demographic details, clinical, radiological and scintigraphic findings of the patients are summarised in Table 6.5. The patients, whose median age was 59.8 years, had been on dialysis for a median duration of 6.3 years, with 97.7% of that dialysis time spent on CAPD. Six patients (46.2%) had no clinical, radiological or scintigraphic evidence for DRA. Of the remaining 7 patients (53.8%), positive scintigraphy alone was documented in 3 cases, and clinical features of DRA in the remaining 4 cases were confirmed scintigraphically (Figure 6.11) in all cases, and radiologically in only one (Figure 6.12). Carpal tunnel syndrome was the most common presenting symptom and only one patient (BJ) needed carpal tunnel decompression. The only patient (AP) to have arthralgia characteristic of DRA had also been dialysed longest at 17.3 years, and he was also the only patient to have radiological features of amyloid bone cysts. Scintigraphically, positive wrist uptake was seen in all patients with a positive scan, whilst the shoulders and knees were affected in 2 cases (BJ & AP). In these 2 cases, the asymmetric intensity in tracer uptake in the wrists corresponded with their clinical symptoms. Total body amyloid load as determined by metabolic turnover studies was small in all affected cases.

Although the 7 patients with DRA tended to be older (59.8 vs 58.8 years) and was on dialysis longer than those without DRA (6.4 vs 5.9 years), the difference was not statistically significant because of the small numbers of patients involved.

Table 6.5 Clinical details and results

Patient	Sex	Age (yrs)	TDD (yrs)	%age dialysis time on CAPD	β2-M (mg/L)	Symptoms	Scans	X-rays
FC	M	73	5.8	96.4	32.5	-	-	-
GI	F	57.3	7.8	94.7	38.1	-	-	-
JR	M	77.6	5.8	100	29.2	-	-	-
SS	F	42.6	6.3	93.3	23.5	-	-	-
YL	F	42.6	6	98.6	47.7	-	-	-
RHS	F	60.2	5.5	97.7	50	-	-	-
MM	M	57.7	6.4	97.4	29	-	Both wrists	-
HR	M	71.8	6.4	96.1	40	-	L wrist	-
DS	M	73	6.6	100	35.6	-	R wrist	-
SC	F	47.8	6	98.6	35.5	Bilateral CTS	Both wrists, R>L	-
STH	F	42.4	6.2	100	31.4	Bilateral CTS	Both wrists, R>L	-
BJ	F	69.8	9	98	23.4	CTS, R only	Both wrists, shoulders (R>L), knees	
AP	M	59.8	17.3	84	36.5	Bilateral CTS, R>L & stiff R shoulder	Both wrists (R>L) & shoulders	Cysts, x3 R wrist, x1 L hip
Median (Interquartile range)		59.8 (48-72)	6.3 (6-6.6)	97.7 (96.1-98.6)	35.5 (29.2-38.1)			

TDD, total dialysis duration

Compared to published figures [156], serum β2-M levels were similar to other CAPD patients and patients on HD with AN69 membranes but lower than those on HD with cuprophane membranes.

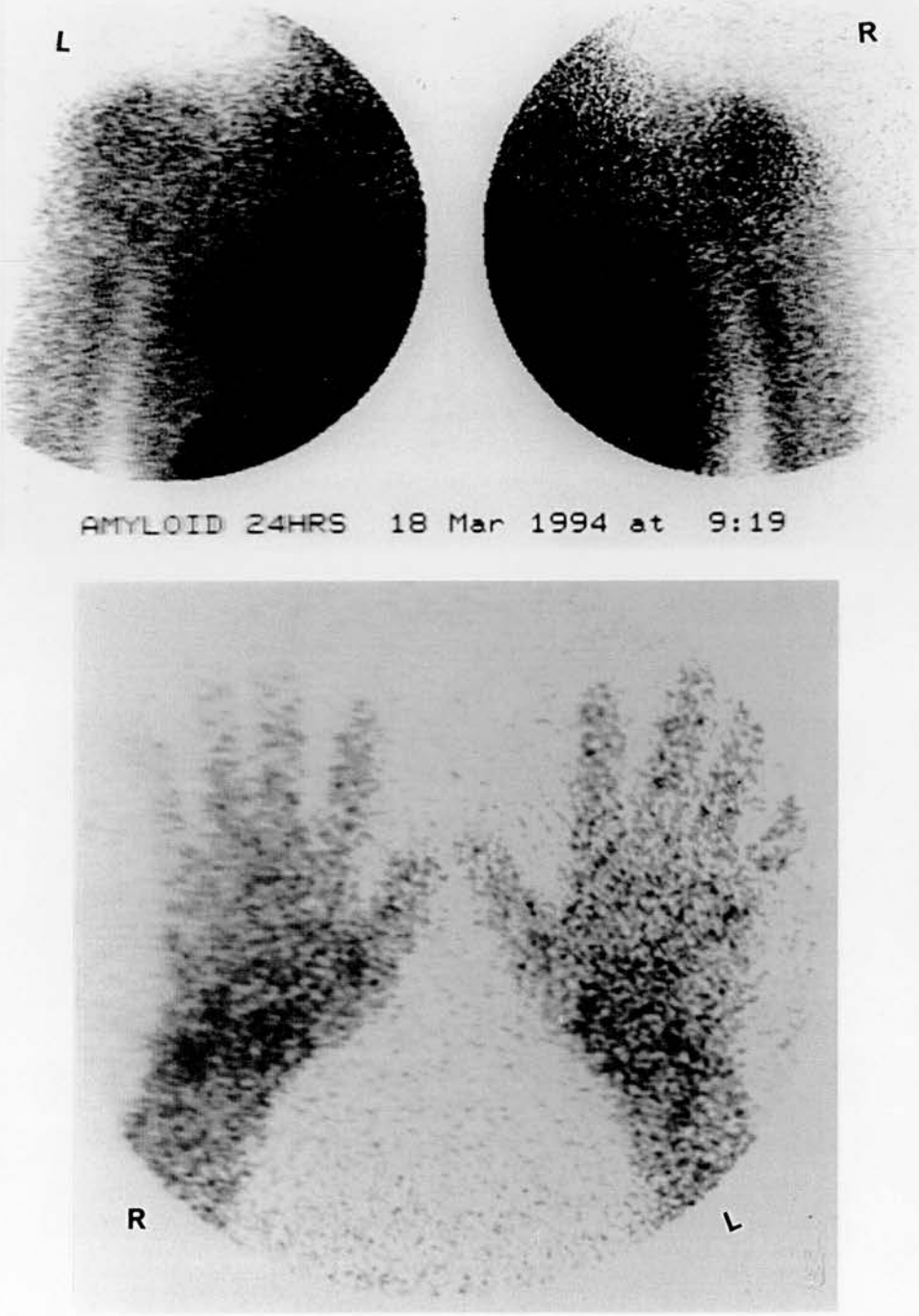

Figure 6.11 ^{123}I-labelled SAP scans 24 hours after injection of tracer in a patient (BJ), demonstrating uptake in both wrists (R>L) and shoulders (R>L).

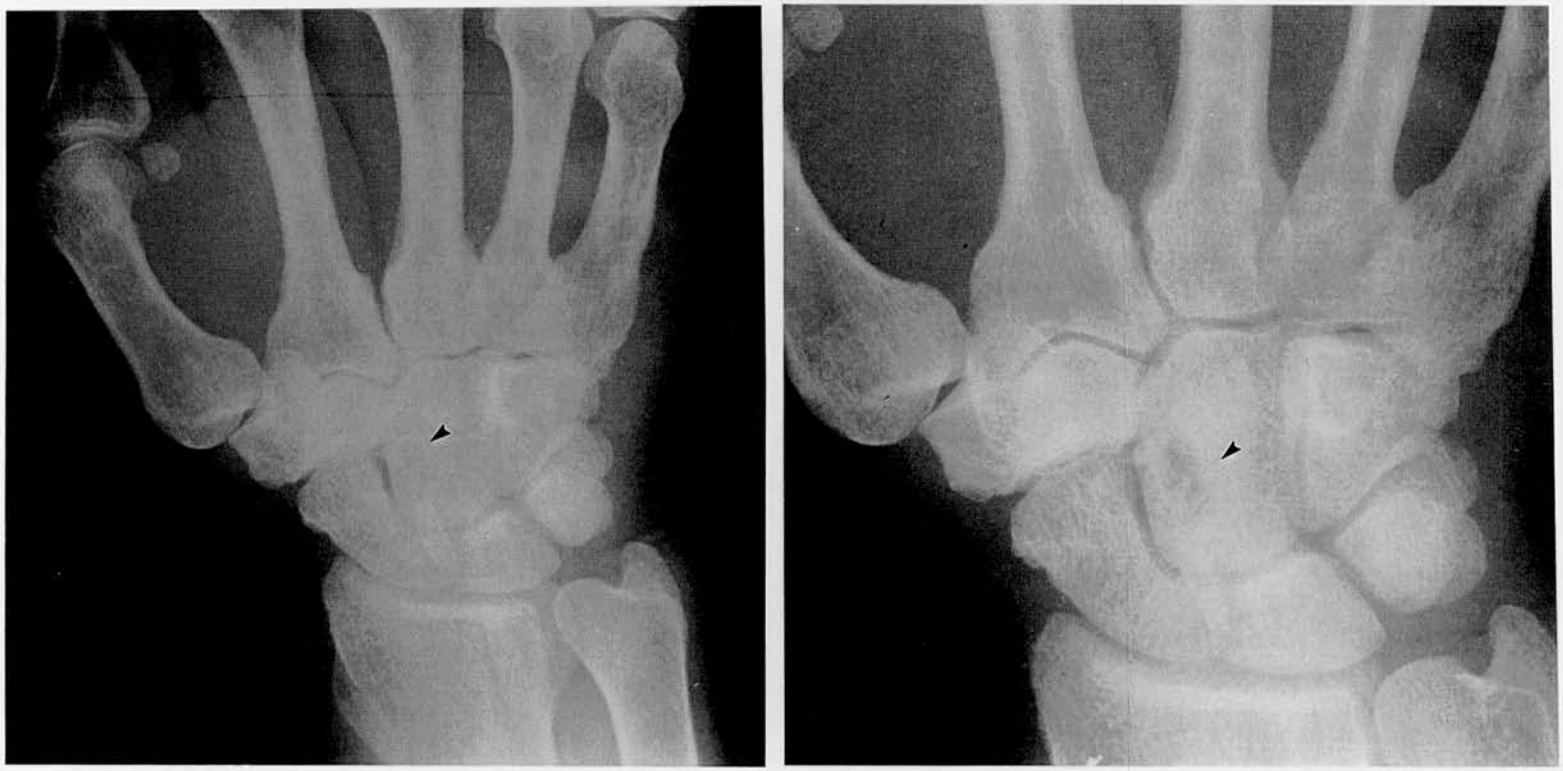

Figure 6.12 Serial X-rays of right wrist of a patient (AP) taken in March 1988 (left) and January 1992 (right), demonstrating increase in size of DRA bone cysts *(arrow)*.

Discussion

Our data confirm earlier observations that DRA may also complicate CAPD [186-188] and, as in HD patients, prevalence of the disease is frequently underestimated clinically and especially, radiologically [183, 191]. Fifty four percent of our patients had scintigraphic evidence for the disease but only 31% were symptomatic and only 1 (8%) had classical radiological features of the disease. In comparison, a post-mortem study of 44 HD patients calculated that 50% of 60 year old patients are likely to have histological evidence for DRA after 5 years of HD [183]. This figure is similar to the scintigraphic findings in our CAPD patients who were broadly matched in age and duration of dialysis, suggesting that prevalence of DRA is comparable between these two dialysis populations.

Clinically, the prevalence of CTS as a feature of DRA was also reported to be similar in the two dialysis dialysis populations in one study [192]. Our finding that 31% of our patients had clinical features of the disease is similar to the 29% of patients in another study who were found to have CTS and/or arthralgias after 4.7 years of CAPD [187]. Comparison between groups of patients in different studies is however fraught with difficulties due to the different diagnostic criteria, limited patient homogeneity and retrospective nature of the studies. One study of HD patients [161] for example, used the need for carpal tunnel decompression as evidence of CTS due to DRA and found that only 5% of their HD patients had clinical features of the disease after 5 years of HD. In contrast, although 31% of our patients had symptoms of CTS, most were only mildly affected with only 1 needing carpal tunnel decompression and only 1 other patient had arthralgia suggestive of DRA after 17 years of dialysis. Therefore, although the disease may be equally prevalent in CAPD populations, clinical expression of the disease may be different, with apparently less severe symptoms in affected patients in our study. This interesting observation suggest that other factors such as dialysis modality are important in determining disease expression. We [13, 191] have previously confirmed earlier observations [159, 167] that symptoms of DRA may resolve rapidly within hours of a successful transplant and these symptoms may also return rapidly when the patient is put back on HD, even if they remain on steroid

therapy, suggesting that HD, perhaps through products of bioincompatible membrane reactions, are important in determining clinical expression of the disease. This is further supported by reports of improvement and even resolution of symptoms of DRA following conversion from HD with bioincompatible cellulose membranes to the more biocompatible synthetic membranes [175, 193] and following a change in dialysis modality from HD to CAPD [194].

Age and duration of dialysis [13, 161] therefore remain the two most important risk factors for the development of DRA for which the only effective treatment is restoration of tubular renal function by renal transplantation [13, 159, 191]. The potentially beneficial effect of HD with more biocompatible membranes in reducing prevalence of DRA [161, 173] has not been supported in other studies [195, 196] and is likely to be evident only if such membranes are used early and exclusively throughout dialysis [1, 13]. Despite clearance of small molecular weight proteins resulting in lower serum β2M levels and the perfect biocompatibility of the peritoneal membrane in CAPD patients [189, 190], the prevalence of the disease is not reduced compared to HD patients, a finding supported by other studies [192]. However, the long term effect of CAPD on the development and progression of DRA compared to HD remains to be evaluated by a prospective controlled study which will require a large number of patients because of the likely high drop-out rate due to renal transplantation and conversion of dialysis modalities because of technical failure or peritonitis. Finally, the suggestion from our observations and from other published studies that the likelihood of developing symptoms in association with the articular amyloid deposits may be modulated by dialysis modality or the use of biocompatible membranes in HD will need to be confirmed.

Chapter 7 - Primary localised orbital amyloidosis due to deposition of the immunoglobulin gamma heavy chain CH3 domain

INTRODUCTION

Amyloidosis [1-3, 11] is a disorder of protein metabolism in which normally soluble proteins are deposited in the extracellular space in the form of abnormal fibrils with characteristic ultrastructural morphology and tinctorial properties. Many different proteins can form amyloid fibrils in various amyloid syndromes, and 15 different precursors have been identified to date. The deposits also always contain sulphated glycosaminoglycans, some of which are tightly bound to the fibrils, and a non-fibrillar glycoprotein, amyloid P component, which is derived from and identical to the normal plasma protein, serum amyloid P component. Amyloid deposits can occur in any tissue and may be widely distributed systemically or localised to specific focal areas. They cause disease principally by occupying space and disrupting normal structure and function.

Primary localised orbital amyloid [197-199] is an extremely rare condition in which the deposits are confined to the orbit and occur in the absence of amyloidosis elsewhere. There is usually no antecedent orbital disease. Most patients present with a mass in the anterior orbit or with proptosis. Eye movement may be restricted either by the mass effect of the amyloid or by involvement of the extraocular muscles. Inflammatory changes and loss of visual acuity are rare. The condition is usually slowly progressive.

The eye and orbit may be involved in systemic amyloidosis, for example vitreous amyloid due to variant transthyretin in familial amyloid polyneuropathy, corneal deposits due to variant gelsolin in lattice corneal dystrophy, and amyloid deposits in eyelids and dermal vessels causing periorbital bruising in systemic monoclonal immunoglobulin light chain (AL) amyloidosis [1]. However the fibril protein in primary

localised orbital amyloidosis has not been identified, although it is noteworthy that the deposits are commonly infiltrated with plasma cells, lymphocytes and foreign body giant cells. The most common form of localised amyloidosis in other tissues, including the skin, respiratory and urogenital tracts, is immunoglobulin-derived and associated with non-malignant local clonal B cell/plasma cell proliferation.

Until recently the biochemical identification and characterisation of amyloid fibril proteins has been hindered by the need for appreciable amounts of material, usually only obtainable at autopsy. However it is now possible to analyse picomolar quantities of small proteins and peptides, and we report here such analysis of the traces of amyloid which were extracted, using a specially modified technique, from a diagnostic biopsy in a case of primary localised orbital amyloid.

MATERIAL AND METHODS

Patient

R.B., a 73 year old lady, presented in 1992 with a one year history of right proptosis and more recent difficulty looking to her right. She was blind in her left eye from a perforating eye injury as a child but was otherwise well with no other relevant past medical history. On examination her visual acuities were: right 6/9, left not perceiving light; with her right eye she could see 17/17 with Ishihara colour plates suggesting that there was no optic nerve compression. There was periorbital oedema on the right side with 2 mm proptosis, 6 mm medial deviation and 3 mm inferior deviation of the globe (Figure 7.1). The right eye showed no abduction, minimal adduction and downward gaze, but only slightly limited upward gaze. The pupil reacted briskly to direct light and fundoscopy was normal.

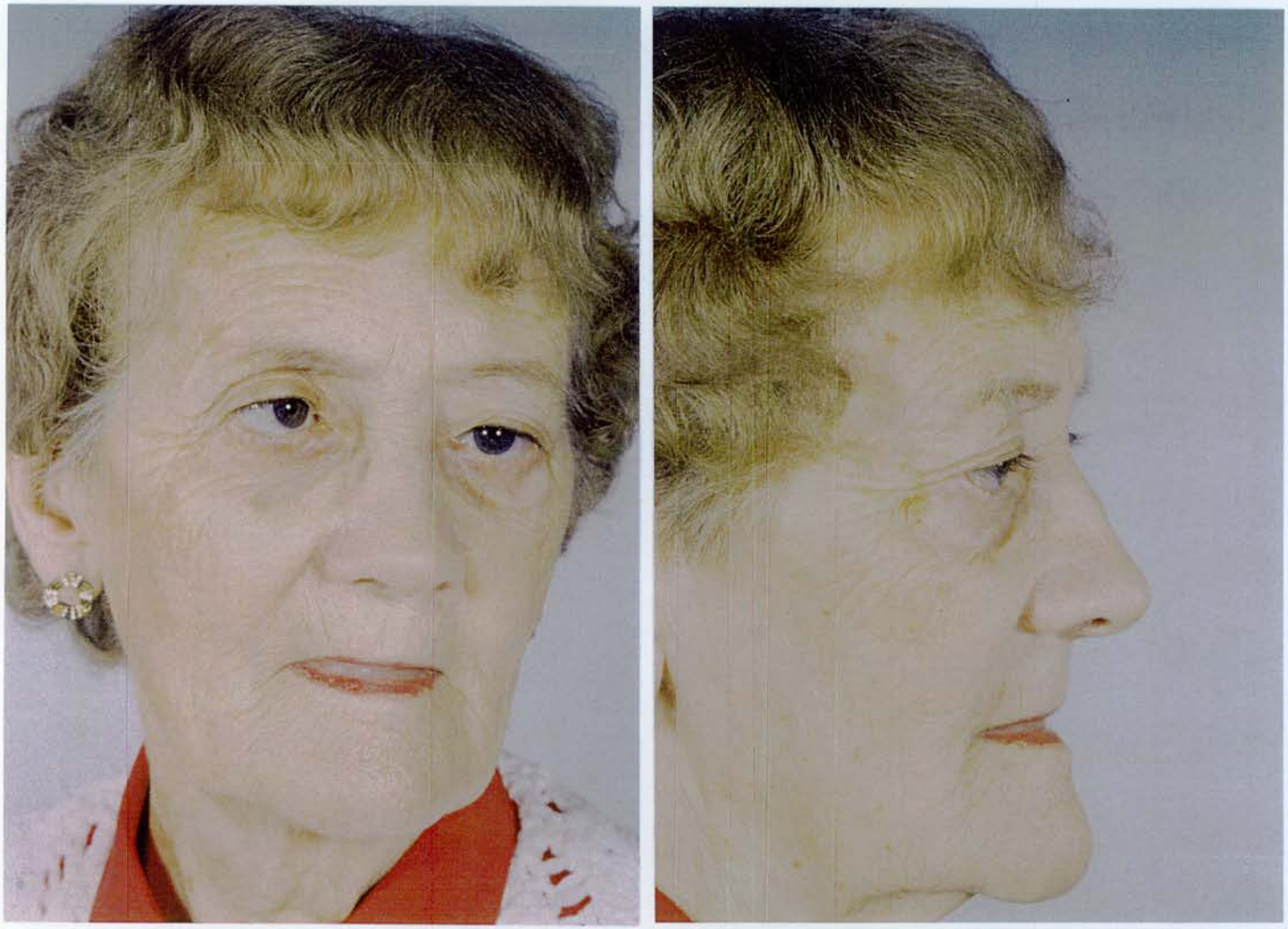

Figure 7.1 Frontal *(left)* and lateral *(right)* views showing periorbital oedema, proptosis and deviation of the right globe.

Clinical investigations and results

Computerised tomography showed a mass in the upper outer quadrant of the right orbit, extending behind the globe (Figure 7.2). Biopsy performed through the orbital septum yielded connective tissue heavily infiltrated with amyloid, demonstrated histologically by Congo red staining [4]. Some plasma cells and lymphocytes were seen but immunohistochemical staining with antisera to κ and λ light chains provided no evidence for clonal restriction. No other pathology was seen. Full blood count, serum biochemical profile, erythrocyte sedimentation rate, serum C-reactive protein, and electrophoresis of serum and urine proteins were all normal. Bone marrow aspirate and trephine biopsy were both cytologically normal, with no evidence for an abnormal B cell clone, and no immunoglobulin gene rearrangement was detected in either the bone marrow aspirate or peripheral blood. Skeletal X-ray survey was normal. Scintigraphy with ^{123}I-labelled serum amyloid P component [58, 59] was normal with no evidence for systemic or visceral amyloidosis.

Extraction and isolation of amyloid protein

The biopsy from the orbital lesion, total mass ~ 3 mg, was stored frozen at -70°C. Since histologically the bulk of the tissue was amyloid a portion (~ 1 mg) was analysed directly by electrophoresis in SDS 8-18% gradient PAGE (ExcelGel™ Pharmacia Biotech, St Albans, Herts, U.K.), in the hope that the amyloid fibril protein would be the major species present and could then be identified by protein sequencing after electrotransfer to a suitable membrane.

The standard water extraction method [8] for isolation of amyloid fibrils from tissue involves numerous preliminary homogenisations in saline, during which, in our experience, significant losses of fibrils may occur. In order to maximise recovery from the ~ 2 mg of tissue available it was initially homogenised in 0.5 ml of distilled water to extract the fibrils immediately. After centrifugation at 15,000 *g* for 10 min and separation of the supernatant, the process was repeated twice more and the

supernatants were pooled together. The amyloid fibrils were then selectively precipitated by adding sodium chloride to a final concentration of 0.2 mol/l, leaving essentially all other components of the water extract in solution in the saline. The suspension was centrifuged at 15,000 *g* for 10 min and the presence of amyloid fibrils was confirmed by drying 10 µl of resuspended material on a poly-L-lysine coated slide, fixing with 10% formalin/90% ethanol and staining with Congo red [4]. The protein composition of the fibril pellet was analysed by SDS 8-18% gradient PAGE.

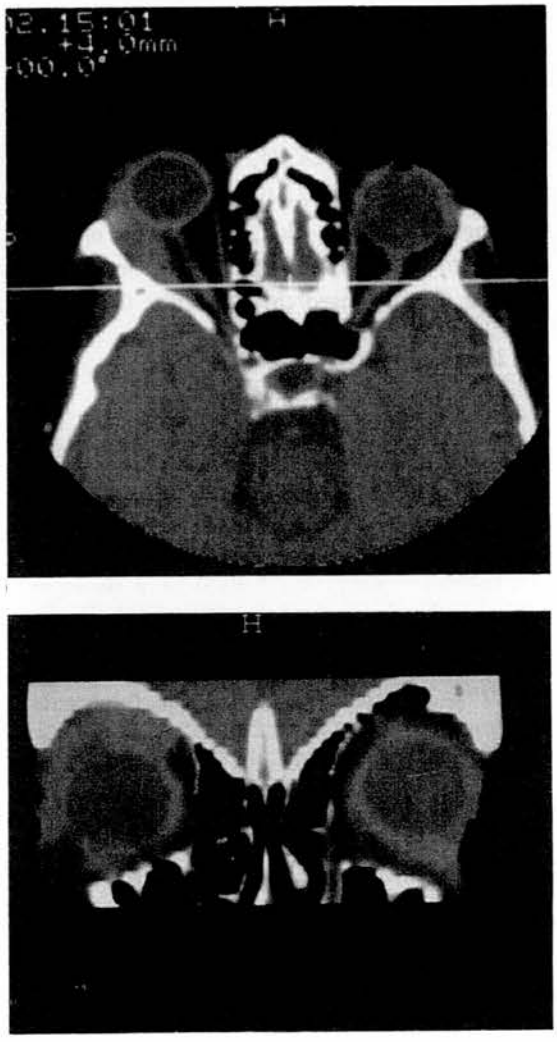

Figure 7.2 Transverse *(top)* and coronal *(bottom)* computerised tomographs of the orbits.

Sequence analysis

A fragment of the whole biopsy was run on SDS 8-18% gradient PAGE and the separated proteins transferred by electroblotting to PVDF membrane. After staining with Coomassie blue the major band at ~ 14 kDa was excised and sequenced from the *N*-terminal on an Applied Biosystems (Foster City, CA, U.S.A.) 477A instrument [83]. The whole purified amyloid fibril preparation was also sequenced directly on the same instrument, and then repeated with inclusion of proline cleavage cycles [83]. Protein mass of the fibril preparation was determined by time-of-flight laser desorption spectrometry in a Finnigan-MAT Lasermat (Finnigan-MAT, Hemel Hempstead, Herts, U.K.), which was calibrated by inclusion of insulin on an α-cyano-4-hydroxycinnamic acid matrix.

RESULTS

Whole orbital amyloid tissue

SDS PAGE analysis of a whole fragment of the biopsy revealed 14 different bands (Figure 7.3) the most abundant of which, running at ~ 14 kDa, was expected to represent the amyloid fibril protein because Congo red staining showed that it was heavily infiltrated with amyloid. However, partial amino acid sequencing of this band identified the sequences of haemoglobin α and β chains. We have obtained similar results with other amyloid-containing biopsies (unpublished observations), even when no blood was visible by naked eye inspection.

Isolation of amyloid fibrils

The modified water extraction procedure yielded amyloid fibrils as shown by Congo red staining and the pathognomonic apple green birefringence when viewed with high intensity cross polarised light (Figure 7.4).

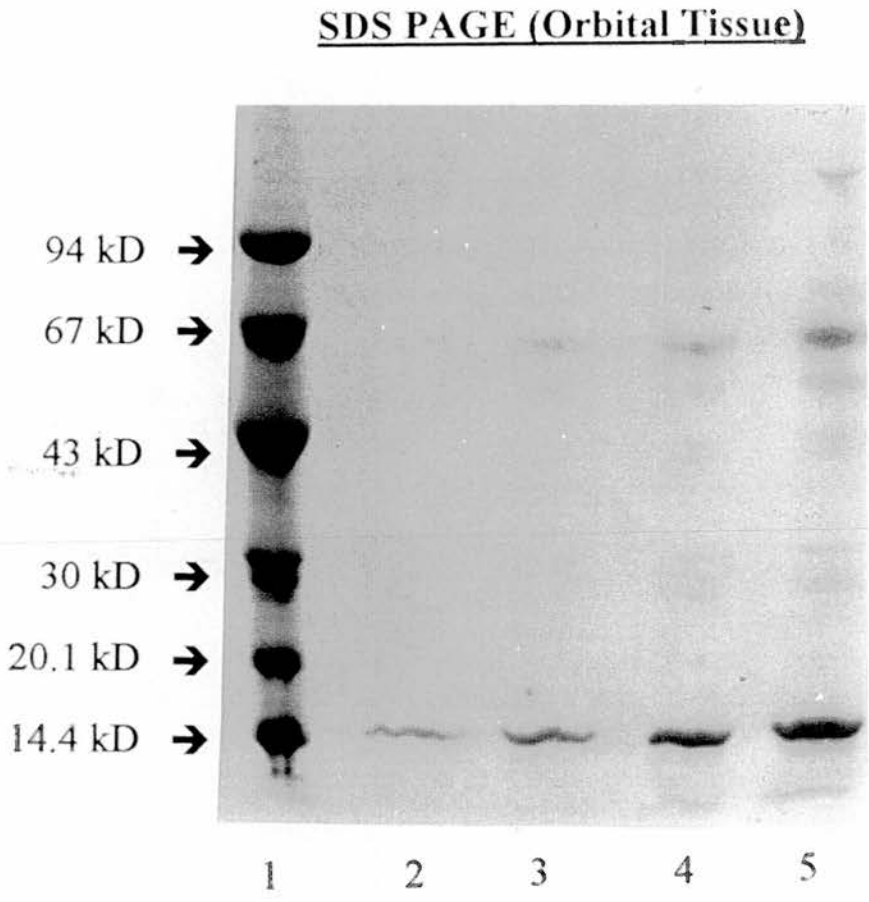

Figure 7.3 SDS 8-18% PAGE analysis of whole orbital amyloid biopsy tissue: Lane 1, molecular weight markers; lanes 2-5, increasing loadings of the reduced denatured tissue sample.

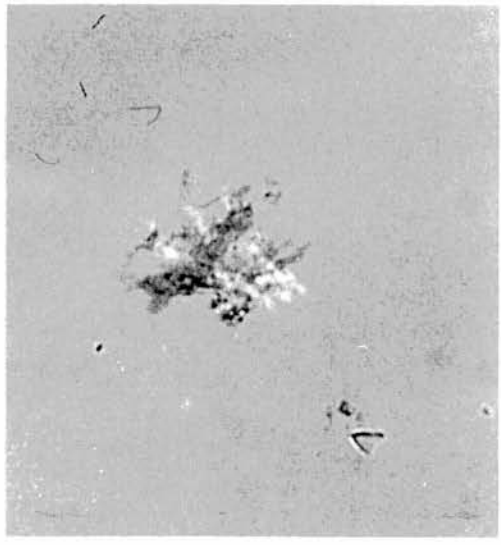

Figure 7.4 Extracted orbital amyloid fibrils stained with Congo red and viewed in polarised light, showing the pathognomonic birefringence of amyloid.

Identification and characterisation of the amyloid fibril protein

SDS PAGE analysis of the extracted amyloid fibrils revealed only one band migrating at ~ 6 kDa (Figure 7.5); the same band was also present in the SDS PAGE of the whole biopsy (Figure 7.3, lane 5). Automated amino acid sequencing of this protein revealed that the 9 *N*-terminal residues of the protein corresponded to the known sequence in the *N*-terminal region of the third constant domain (CH3) of the γ1 heavy chain of IgG1 subclass (positions 278 to 286) and also of the γ4 heavy chain of IgG4 subclass (residues 275 to 283) (Figure 7.6). It was not technically possible to obtain any further sequence from the extremely limited amount of protein available.

The mass of the purified amyloid fibril subunit protein by time-of-flight analysis was 6125 Da, whilst the predicted mass of the whole γ1 CH3 domain from residue 278 is 6169.9 Da and that of the γ4 CH3 domain from residue 275 is 6214.9 Da.

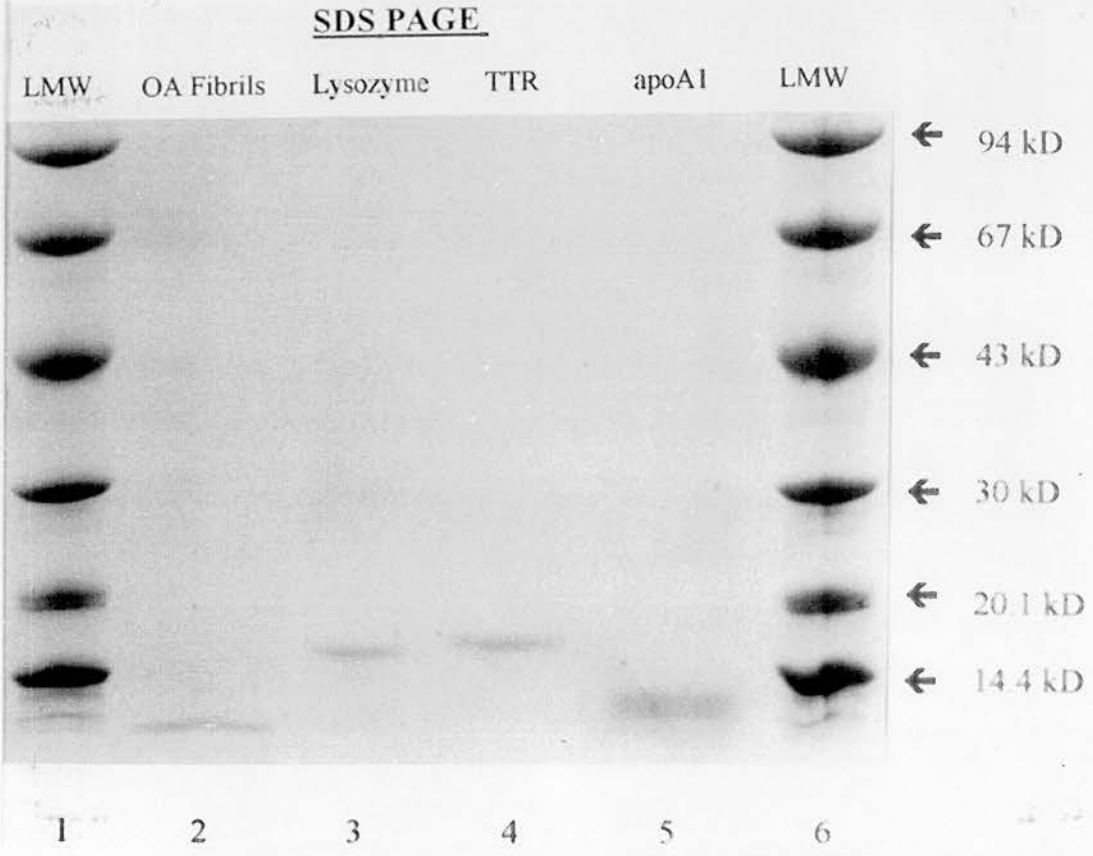

Figure 7.5 SDS 8-18% PAGE analysis of extracted amyloid fibrils: Lanes 1 and 6 contain molecular weight markers; lane 2, isolated orbital amyloid fibrils; lane 3, lysozyme amyloid fibrils [21]; lane 4, purified human transthyretin; lane 5, purified subunit from apolipoprotein AI amyloid fibrils [19].

```
                                        XTPPVLDSDG
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
 250        260        270        280        290
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
 300        310        320        330

                                        XTPPVLDSDG
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVD
    250        260        270        280        290
KSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
    300        310        320
```

Figure 7.6 *N*-terminal amino acid sequence of the orbital amyloid fibril protein aligned with the sequences of the CH3 domains of γ1 (above) and γ4 heavy chains (below).

DISCUSSION

Monoclonal immunoglobulin light chains were the first amyloid fibril protein to be identified by protein sequencing [9] and this type of amyloidosis, designated AL, is a well recognised condition complicating about 15% of cases of multiple myeloma and also a significant proportion of other monoclonal B cell disorders [1, 200]. The actual amyloid fibril protein in all cases of AL amyloidosis in which it has been sequenced consists of part or all of the variable domain, together, in some cases, with part or, rarely, all of the rest of the light chain [1, 200]. Since normal polyclonal light chains and most monoclonal ones do not form amyloid it is generally accepted that unique individual sequences of the amyloidogenic monoclonal light chains are responsible for fibril formation [2, 3]. This concept is strongly supported by the fact that λ light chains are more commonly involved in AL amyloid than κ chains, although the latter predominate among both normal immunoglobulins and monoclonals in general [2, 3, 200]. Furthermore monoclonal gammopathy in which light chains of the VλVI

subgroup are involved is almost always complicated by amyloidosis. Finally when Bence-Jones proteins from myeloma patients with amyloid are injected into mice they form amyloid deposits whereas Bence-Jones proteins from myeloma patients without amyloid do not [201].

In contrast immunoglobulin heavy chains and their fragments generally seem to lack whatever primary and higher structure is required for amyloid fibril formation *in vivo*. In 1974 a case of systemic amyloidosis associated with heavy chain disease was reported in which the γ heavy chains formed amyloid-like fibrils *in vitro* [202], but only two definite cases of heavy chain-derived amyloidosis have been confirmed by sequencing. The first such patient had amyloid in the spleen, liver, kidneys and heart, a pattern of distribution characteristically seen in AL amyloid, but the fibrils consisted of an intact heavy chain variable domain joined to a complete third constant domain of γ1 subclass [203]. The second case of heavy chain amyloid also had a typical presentation for AL amyloid with a monoclonal serum IgGκ protein identified by heavy chain sequence as IgG1 subclass, Bence-Jones proteinuria, and amyloid in the spleen and kidneys [204]. The amyloid fibrils contained just the diversity segment of the heavy chain variable region, V_H-D. These cases have been designated as AH type amyloidosis.

The present patient with primary localised orbital amyloidosis is the third case of AH, immunoglobulin heavy chain-associated amyloid. The fibril protein is derived from the CH3 domain of a γ heavy chain of either γ1 or γ4 subclass, without any apparent contribution by the variable domain. This is a unique and exceptional finding since in all cases of both heavy and light chain amyloid characterised so far the fibril proteins included at least part of the variable domain [200, 203, 204]. Unfortunately insufficient material was available to permit further sequencing in order to identify either the γ subclass of origin or detect the presence of variant residues which may have been responsible for amyloidogenicity. Although the mass of the fragment suggests that virtually the whole domain from the identified *N*-terminal residue was present, the precision of laser desorption mass analysis (~ 0.5%) was insufficient to support speculation about the presence of variant residues, *C*-terminal raggedness or

glycosylation. However given the rarity of heavy chain amyloidosis it is possible that some unusual structural feature may be present.

The localised nature of the amyloidosis in this case, the absence of any sign of a systemic monoclonal gammopathy, and the plasma cell/lymphocyte infiltrate in the lesion, strongly suggest that these cells were the source of the heavy chain fragment deposited as amyloid. It is not clear whether the amyloidogenic protein was derived by proteolysis of a larger precursor or even an intact γ chain, or whether it represents an abnormally spliced product generated by disturbed immunoglobulin gene rearrangement in an autonomous clone. Unfortunately no tissue was available for immunoglobulin gene rearrangement studies. Plasma cell infiltration has been reported in all other cases of primary localised orbital amyloidosis suggesting that these are also immunoglobulin-related, but it remains to be determined whether they are of light or heavy chain origin.

Chapter 8 - Treatment of amyloidosis

Introduction

The past 25 years have seen major advances in our understanding of the pathogenesis and natural history of amyloidosis. Although specific treatment of amyloidosis is not yet available, the demonstration that amyloid deposits are derived from circulating or locally produced precursor proteins that are abnormal in structure or concentration or both have led to the development of treatment strategies aimed at reducing the supply of precursor proteins in the hope that disease progression can at least be halted. The benefit of such therapy have been demonstrated by subsequent improvement in organ function, histological and scintigraphic evidence for amyloid regression, and most importantly, improvement in morbidity and survival. At the same time, major advances have been made in the treatment of renal and heart failure which are the main causes of death from amyloidosis. The development and availability of dialysis and latterly, organ transplantation means that prognosis may be considerably improved in systemic amyloidosis. Major therapeutic advances have therefore been made over the past 20 years since one of the first attempts at treating amyloidosis was made with DMSO [205].

Prospective evaluation with ^{123}I-SAP scintigraphy

The effect of treatment of amyloidosis on organ function may be monitored by certain laboratory markers such creatinine and 24 hour urinary protein excretion in renal amyloid and liver function tests in hepatic amyloid. However, organ amyloid load corresponds poorly with organ function, changes of which provide no useful information on the physical effect of treatment on amyloid deposits. Prospective evaluation by repeat biopsies have demonstrated regression of amyloid following

treatment [46, 206, 207] but invasive biopsies are unlikely to have a role in the routine monitoring of treatment and in any case provide only local qualitative information that is subject to sampling error (Table 8.1). Serial radiology (Figure 6.7) may be used but only for following bone cysts due to DRA [26, 159]. The only investigation that have been used effectively and reproducibly in monitoring systemic amyloidosis is serial ^{123}I-SAP scintigraphy which have confirmed previous suggestions that amyloid deposits are in a state of dynamic turnover and may be mobilised and redistributed [208] or regress [46, 206, 207]. Response to therapy with scintigraphic evidence for regression have been demonstrated in AL [47, 48, 185] (Figure 8.1), AA [12, 49] (Figure 8.2), β2-M [165] (Figure 6.9 & Figure 6.10) and hereditary [50] amyloidosis. In none of these patients have amyloid progressed despite effective treatment of underlying disease and neither was there spontaneous regression without treatment.

Table 8.1 Comparison of histology and SAP scintigraphy in amyloidosis

Histology	SAP scintigraphy
Microscopic sampling	Whole body survey
Permits immunohistochemical typing	Distribution of amyloid may be diagnostic of type
Poorly quantitative	Permits quantification
Invasive	Serial monitoring
Widely available	Restricted availability at present

Management

A feature of the abnormal protein metabolism seen in amyloidosis is the imbalance between rate of deposition of fibril precursor proteins and the relative but not

complete resistance of fibrils to degradation and mobilisation. There are, at the moment, no treatment available that may promote or accelerate degradation of fibrils. Specific treatment of amyloid is therefore aimed at reducing supply of precursor (Table 8.2) in the hope that fibril deposition and disease progression may be slowed down or even halted, thereby favouring mobilisation of amyloid. Clinical evidence that this occurs and may be accompanied by regression of amyloid have been confirmed scintigraphically [12, 47, 49, 50, 165, 185] and histologically [46, 206, 207].

Reduction in supply of precursor proteins is achieved in AL amyloid by suppressing plasma cell synthesis of clonal precursor immunoglobulin light chains, and in AA amyloid by suppressing the stimulus for hepatic synthesis of precursor SAA proteins. However, in DRA, reduction in supply of precursor β2-M is achieved by improving dialysis efficacy so that clearance of β2-M is increased. In both DRA and hereditary transthyretin amyloidosis, organ transplantation restores normal protein metabolism such that renal transplantation leads to normal β2-M clearance whilst the source of systemic variant TTR is eliminated following a successful liver transplant. Until treatment aimed at degradation and mobilisation of amyloid is developed, these strategies remain the most effective treatment available for amyloidosis.

In patients who are already affected by the disease, supportive therapy remains a critical component of their management, delaying target organ failure, maintaining quality of life and prolonging survival whilst specific therapy is instituted. Such supportive therapy may in some cases 'buy time' and allow patients to live long enough to see the benefit of chemotherapy which may be delayed [47].

Patients with renal amyloid are acutely sensitive to volume changes because stiff and poorly reactive amyloid-laden glomerular vessels are unable to respond sufficiently to changes in blood volume and pressure. Renal failure is often irreversible in amyloid kidneys and maintenance of volume status and blood pressure especially perioperatively is therefore critical. Proteinuria and even nephrotic syndrome may respond to colchicine or treatment of the underlying disease although nephrectomy may rarely be necessary [209]. Clinically significant cardiac amyloid is characteristically manifested by restrictive cardiomyopathy whereby maintenance of cardiac output is dependent on

high filling pressures and vasodilators should therefore be avoided and diuretics used with caution in these patients. There may be increased sensitivity to digoxin because of binding to amyloid fibrils. Sudden death due to arrythmia is common and prophylactic pacing may therefore be necessary.

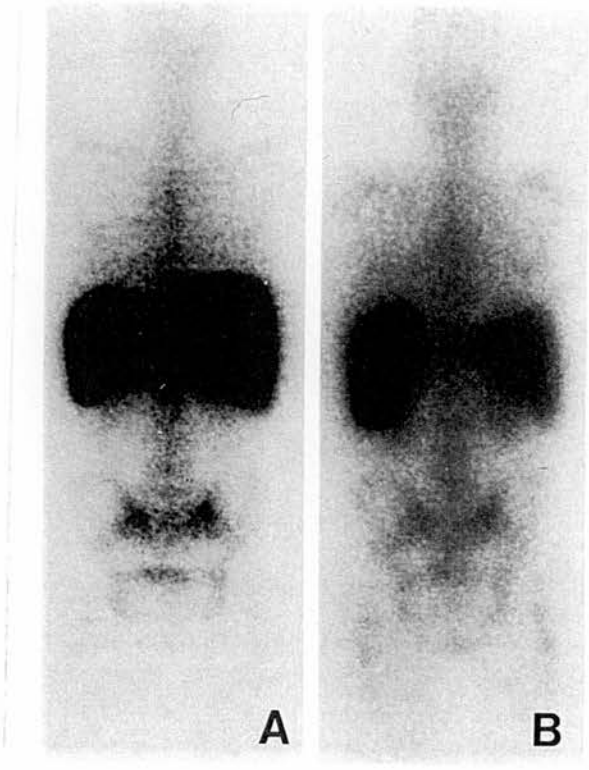

Figure 8.1 Regression of AL amyloidosis. Serial posterior whole body ^{123}I-SAP scintigraphs of a man with AL amyloidosis presenting with hepatomegaly and massive elevation of liver alkaline phosphatase. Initially *(A)* there was extensive uptake in the spleen, liver (largely obscuring renal deposits) and bone marrow. Remarkably, a second scan *(B)* performed 6 months later after 4 cycles of 'VAD' chemotherapy shows major regression of the liver deposits, associated with near normalisation of alkaline phosphatase, but little change in the splenic and bone marrow amyloid.

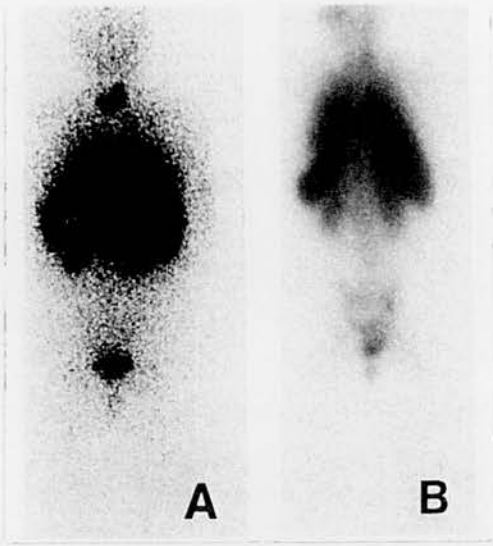

Figure 8.2 Regression of AA amyloidosis. Serial posterior whole body ^{123}I-SAP scintigraphs of a young woman with JRA complicated by AA amyloidosis. Scans at presentation with nephrotic syndrome *(A)* show intense uptake of labelled SAP into extensive amyloid deposits in the liver, spleen, kidneys and thyroid gland. Treatment with chlorambucil induced a complete remission of inflammatory disease activity and was followed by the disappearance of proteinuria; repeat SAP scan 3 years later *(B)* indicates substantial regression of amyloid with only minor splenic adrenal deposits remaining. The remainder of the image is blood pool background which is increased when specific uptake into amyloid is reduced, and radioactive breakdown products in the bladder.

In patients with renal failure, supportive treatment with dialysis [210-213] or renal transplantation [214-217] may considerably improve prognosis and in patients with cardiac amyloid, heart transplantation [47, 218] may be life saving but optimal management of these patients must include specific therapy aimed at reducing supply of precursor proteins. Parenteral nutrition may be essential in malnourished patients who present with severe systemic disease and are at risk of pressure sores and infections because of immobility and impaired bladder and bowel function. Dry gritty eyes due to keratoconjunctivitis sicca, usually seen in AL or hereditary amyloid may be relieved by artificial tears and should also be regularly examined for infection and corneal abrasions or ulcers.

However, as with many diseases, prevention may be possible, is cost effective, and remains the ultimate goal of primary care. The success of preventive strategies is probably best illustrated by the changing pattern of AA amyloidosis which followed the introduction of antibiotics such that chronic inflammatory diseases have replaced chronic infectious diseases as the most common cause of AA amyloidosis, at least, in the developed world [219]. This was followed by the demonstration that development of AA amyloid due to FMF may be prevented altogether if colchicine is given early and in adequate doses [220]. DRA, now a major cause of morbidity in long term dialysis populations, may be prevented, most effectively, by an early renal transplant [13, 26].

Preventive measures, however, can be implemented only if patients at risk can be identified. Hence in AA amyloid, patients who might be at risk should have their CRP and SAA proteins measured regularly so that appropriate treatment can be instituted early and its effectiveness monitored. The risk of developing DRA may be reduced by identifying those most at risk so that limited resources with expensive but more efficient dialysis modalities can be offered to those most likely to benefit. Similarly in hereditary transthyretin amyloidosis, optimal timing of liver transplantation may prevent disease progression and irreversible organ damage in carefully selected patients.

Table 8.2 Reducing the supply of fibril precursors in systemic amyloid

DISEASE	AIM OF TREATMENT	EXAMPLE OF TREATMENT
AA amyloid	Suppress acute phase response	Immunosuppression in rheumatoid arthritis, juvenile chronic arthritis (chlorambucil). Colchicine for familial Mediterranean fever, even if clinical episodes not fully suppressed. Surgery for osteomyelitis, and rare cytokine-producing tumours
AL amyloid	Suppress production of monoclonal immunoglobulin light chains	Chemotherapy for myeloma and monoclonal gammopathy
Hereditary amyloidosis	Eliminate source of genetically variant protein	Orthotopic liver transplantation for familial amyloid polyneuropathy associated with transthyretin variants
Dialysis-related amyloidosis	Reduce plasma concentration of β2–M	Renal transplantation

Treatment of the amyloidosis syndromes

Primary (AL) amyloid

Primary AL amyloid is a progressive systemic disease with a poor prognosis. In three series [16, 53, 221] comprising almost 500 cases of AL amyloidosis, the median survival was 14.7 months or less, and may, [53, 221] or may not [222] be worse, if associated with multiple myeloma. Most deaths are cardiac-related and if CCF is a feature of the disease, median survival is reduced to 6.5 months.[16, 53, 223]

The precursor protein of AL amyloid is derived from monoclonal immunoglobulin light chains synthesised by plasma cells. Treatment of AL amyloid is therefore aimed at suppression of the abnormal clone of plasma cells, usually with melphalan, an alkylating agent often used in the treatment of myeloma, in the expectation that supply of precursor light chains will be reduced or stopped, enabling disease progression to be halted. Until recently, the beneficial effect of such treatment which have included histological [46, 207] or scintigraphic [47, 185] evidence for regression of the disease, was confined to isolated but numerous case reports [46, 224-240]. These reports have also included the use of colchicine [225, 241-243] which have been used advantageously in other types of amyloidosis.

Several clinical trials have now been published (Table 8.3), comparing the use of melphalan, prednisolone and colchicine [223, 244-247]. Initial trials with melphalan and prednisolone reported that although there may be an improvement especially with resolution of proteinuria or nephrotic syndrome, there was no beneficial effect on survival [223, 244]. However patient numbers were small and treatment duration shorter than subsequent studies reporting more encouraging results [246, 247]. Improved survival in patients treated with colchicine alone reported by another study used untreated historical controls for comparison [245]. However, poor survival in the control group in this study was probably due to a significantly larger proportion of patients with CCF, and subsequent studies have failed to demonstrate a clear benefit in patients treated with colchicine alone [244, 247]. In fact, in a large crossover study in

1985 [244], there was no beneficial effect on survival in patients treated with colchicine despite the use of maximum doses that could be symptomatically tolerated (median 1.5 mg/d). In this study, patients treated with melphalan and prednisolone survived significantly longer, but only in the subgroup of patients who remained on one treatment alone without crossing over, which was allowed after 6 months if there was evidence for progression of the disease. More patients in the colchicine group crossed over to have melphalan and prednisolone added to the treatment and when compared in aggregate, the melphalan and prednisolone group survived longer (25.2 months vs 18 months), but the difference was not significant (p=0.23). Treatment regimes used in this and other studies are summarised in Table 8.4.

Since then, a further study from the same group but involving larger numbers of patients and longer duration of therapy with melphalan and prednisolone have identified a subset of patients who derived substantial survival benefit related to response [246]. As with the previous studies, patients were divided into 4 smaller groups according to the main organ system clinically affected (nephrotic syndrome, CCF, hepatomegaly or neuropathy). Response to treatment was most likely in patients with nephrotic syndrome and least likely in patients with neuropathy. Median time to response was 11.7months. Survival which was shortest in patients with CCF, was substantially prolonged at 89.4 months in the 18% of patients who responded to treatment compared to 14.7 months for the non-responders.

There are several features common to all these studies. The most common presenting manifestation of AL amyloid was nephrotic syndrome, a cardiac event was the main cause of death, and the most important predictors of a poor prognosis was presence of CCF, multiple organ involvement, renal failure and weight loss [223, 244-247]. The prognosis of AL amyloid remains poor although encouraging results have now been achieved following treatment with melphalan and steroids. There is evidence to suggest that poor response to therapy may be related to inadequate dose and duration of treatment [246]. Due to variations in gastric absorption with melphalan, adequate absorption and delivery should be confirmed by increasing the dosage of melphalan given in 6 weekly cycles until mid-cycle leukopaenia is achieved. Longest

survivals have been achieved so far with therapy continued for between 18 to 36 months and this has to be balanced against the risk of dymyeloplasia especially with acute leukaemia which increases with increasing duration of therapy. However, the poor prognosis but substantially prolonged survival in patients who respond to treatment suggest that a trial of therapy with melphalan and prednisolone should be given for at least 12 months or longer as some patients take more than a year to respond. Current evidence suggests that no additional benefit is gained by adding colchicine to melphalan and prednisolone [247]. There is also no evidence that melphalan and prednisolone is superior to melphalan alone [246].

Results of chemotherapy may be further improved by early diagnosis and rapid institution of treatment [245]. In this study, mean interval between symptoms and diagnosis, and between diagnosis and treatment, was 15 months and 2.7 months respectively. A shorter diagnosis-treatment interval correlated with significantly improved survival [245]. In the past, many physicians have been reluctant to recommend therapy in AL amyloid because of poor results [235] causing further delay in instituting treatment which will only add to the diminishing chances of survival .

In addition to melphalan, other alkylating agents such as cyclophosphamide may be used, but in patients who fail to respond, vincristine, adriamycin and dexamethasone as recently proposed for resistant myeloma have been used and it is also possible that this more aggressive regime is more likely to induce a quicker response to treatment [248]. This may be important especially in patients who present with CCF and have a median prognosis of 6.5 months and are therefore unlikely to survive long enough to see the benefit of therapy with melphalan and steroids which has a median response time of 11.7 months. In such patients, life saving heart transplantation followed by chemotherapy may be appropriate [47]. Systemic AL amyloid is not necessarily a contraindication to organ transplantation especially in patients with small total body amyloid load and in fact heart [47, 218] and renal [214, 249, 250] transplantation for patients with AL amyloid have been successfully performed with good results. In patients with renal failure, replacement of renal function by dialysis may prolong survival substantially, especially if there is no echocardiographic evidence for cardiac

amyloid [210, 211, 213, 250]. Recently, autologous bone marrow transplantation followed by chemotherapy was described in a patient with AL amyloid associated with a monoclonal gammopathy but the patient unfortunately died 10 weeks post-transplant from CMV pneumonitis [251].

Systemic AL amyloidosis involving the spleen may rarely be accompanied by clotting factor deficiency leading to catastrophic haemorrhage [252]. When this happens, splenectomy may be the only effective way of correcting the clotting defect [253, 254] although there are case reports of improvement in clotting time following chemotherapy with melphalan and steroids [237]. Treatment with alfa-tocopherol (vitamin E) [255] in 16 patients and interferon alfa-2 [256] in 15 patients failed to demonstrate any beneficial effect with no objective evidence for regression of the disease. Median survival which was 19.4 months and 26.3 months respectively was not superior to that reported with other agents used for this disease.

Table 8.3 Clinical treatment trials for AL amyloid

Ref	Year	Number of patients	Treatment Regimes	Duration (median, months)	Survival (Median, months)	Comments
Kyle [223]	1978	55	MP (n=23) Placebo (n=27)	4.5* 3*	13.3** 13.3**	Double-blind study allowing code to be broken so that placebo gp can be converted to MP. *Duration of R_x when code was broken; thereafter 16/27 placebo patients changed to MP. **average of median survivals for 3 subgroups of patients (nephrotic syn, CCF, neuropathy).
Kyle [244]	1985	101	MP±C (n=49) C±MP (n=52)	12 12	25.2 18	Crossover study allowing patients to have MP or C added to original regime if no benefit after 6 months. Survival was significantly better ($p<0.02$) in patients with CCF Rx with MP±C.
Kyle [244]	1985	58	MP (n=41) C (n=17)		16 3	Data for patients who remained on MP or C alone without crossing over. More patients in the MP group survived longer ($p<0.001$).
Coh [245]	1987	82	C (n=53) HC (n=29)		17 6	Prospective study involving historical controls (HC). Proportionally there was larger number of patients with CCF in HC gp ($p<0.001$) which probably account for the poor survival. Females found to have significantly longer survival in this study ($p=0.003$)
Gert [246]	1991	153	MP,R (n=27) MP,NR (n=127)	24-36 24-36	89.4 14.7	Prospective but uncontrolled study. Patients with renal amyloidosis but normal creatinine and no cardiac involvement was most likely to respond whilst patients with neuropathy was least likely to respond. Median interval to response was 11.7 months.
Kyle [247]	1993	219	C (n=72) MP (n=78) MPC (n=69)		8.5 17 16	Prospective study. Crossover between regimens not allowed. Nephrotic syndrome most common manifestation followed by CCF and peripheral neuropathy. Survival superior in MP/MPC groups compared to C.

Table 8.4 Treatment regimes for AL amyloid

Melphalan/prednisolone
6 weekly cycles of melphalan 0.15 mg/kg/day in 2 divided doses for 7 days and prednisolone 0.8 mg/kg/day for 7 days
Leucocyte and platelet counts checked every 3 weeks
Increase daily dosage of melphalan by 2 mg for each 6 week cycle until mid-cycle leucopaenia or thrombocytopaenia occurs
Reduce dose if there is severe leucopaenia or thrombocytopaenia and in renal failure
Minimum duration of therapy should be 12 months
Colchicine
Commence at 0.6 mg twice a day
Dosage then increased by 0.6 mg daily each week until abdominal cramps or diarrhoea occurs
Colchicine then discontinued and resumed at highest dosage that did not produce side effects

Reactive systemic (AA) amyloid

In reactive systemic AA amyloidosis the acute phase protein, serum amyloid A (SAA) is laid down as amyloid. Any visceral organ may be affected but most cases become clinically evident only when the kidneys are involved. Subsequent progression of the disease with declining renal function determines prognosis and indeed, renal failure is the major cause of death in AA amyloidosis [219, 257, 258]. The importance of recognising complications with AA amyloidosis is underlined by the poor prognosis associated with it in diseases such as JRA which otherwise has a good prognosis [259]. More importantly, it may be preventable as clearly illustrated by the changing pattern of AA amyloidosis which, following the introduction and widespread use of antibiotics, have resulted in a diminishing incidence of amyloidosis secondary to chronic infectious diseases such that it has now been replaced by chronic rheumatic diseases as the major

cause of AA amyloidosis in the developed world [219].

Attention has therefore focused on treatment of chronic rheumatic diseases especially with the discovery in the 1960's of alkylating cytotoxic drugs such as chlorambucil that act by suppressing underlying disease activity resulting in most cases to suppression of the associated acute phase response (APR). Reduction of precursor SAA levels achieved may reduce the risk of development of AA amyloidosis, and in patients who already have AA amyloidosis, may lead to mobilisation and regression of amyloid. Several clinical trials have been conducted in an attempt to determine the effect of such treatment on progression of the disease in patients with chronic rheumatic diseases complicated by AA amyloidosis (Table 8.5) [260-264]. All patients in these trials had proteinuria which was the earliest indicator of complications with amyloidosis and also the commonest mode of presentation, whilst most patients had histological confirmation of the diagnosis. The mean interval between presentation with underlying disease and diagnosis of amyloid was 10-16 years. Response to treatment was documented in most cases, the most obvious feature of which was reduction in or even complete resolution of proteinuria/nephrotic syndrome, which may or may not be accompanied by histological [206] or scintigraphic [12, 49] evidence for regression of the disease. However, the most notable and indeed dramatic finding was significantly and in some cases considerably improved survival compared to untreated groups.

The first and most notable of these studies involving 51 patients with juvenile chronic arthritis (JCA) was published in 1977 [260]. Proteinuria was present in all patients at diagnosis but in only 36% after > 3 years of continuous treatment with chlorambucil. Survival was 100% at 5 years and at follow up after 15 years [263], 68% of the treatment group was still alive compared to 100% mortality in the untreated group. Other studies have all also reported a major impact on survival in treated patients. The drugs most commonly used was chlorambucil, followed by cyclophosphamide and podophyllotoxin derivatives.

However, there are at least 2 major factors that may contribute to the vastly improved survival seen with treatment. Firstly, treatment and control groups may not be comparable. Patient numbers were much smaller [260] in the treatment groups

whilst historical controls were sometimes used [261]. More importantly, information on renal function, the most important prognostic indicator, was provided and comparable between the study groups in only one trial [262] which was also the only prospective randomised trial so far. The poorer than expected survival in most of the control groups suggest that proportionally, they may have more patients with renal failure and this was confirmed in at least one study [263].

Secondly, alkylating treatment with chlorambucil was introduced in the 1960's at the same time that HD became increasingly available. It is well recognised that availability of dialysis and latterly, renal transplantation, has improved considerably the prognosis of AA amyloidosis [211, 213, 265], a not unexpected finding since renal failure was and remains the major cause of death. The improved survival in treated patients may therefore be partly attributable to the replacement of renal function by dialysis or renal transplantation. The proportion of patients in both groups that was offered renal replacement therapy is not known and it is also possible that patient selection for treatment and subsequently, dialysis or transplantation, may be biased especially in the non-randomised trials. Indeed, in one trial [266], renal function was normal in all patients in the much smaller treatment group. Finally, the favourable influence of renal replacement therapy on survival is supported by the observation that in patients treated with chlorambucil, survival was superior in those treated after 1980 compared to those treated before 1969 [263] when renal dialysis was only becoming available and renal transplantation was uncommon. The impact of treatment with alkylating cytotoxic drugs on prognosis in AA amyloidosis associated with rheumatic disease can therefore only be accurately assessed by renal survival rather than patient survival.

However, the effect of treatment with alkylating agents on the underlying disease is unequivocal. Control of inflammatory activity may be achieved within 1 year [260], is often accompanied by reduction or resolution of proteinuria, and significantly, may lead to slowing down of the decline in renal function [262, 264]. Apparently improved renal survival compared to untreated controls has in fact been suggested in one study [262]. Progression of amyloid in the kidneys and elsewhere may be halted

and regression may even occur but the effect on renal survival without need for renal replacement therapy remains to be determined.

There have been at least 8 case reports [267-272] on the beneficial use of colchicine on AA amyloidosis due to chronic inflammatory diseases. All but one [269] was manifested by nephrotic syndrome which resolved on treatment with colchicine. Reduction of proteinuria may be achieved within 2 to 3 months of treatment and in 3 cases [267-269] this was accompanied by improvement in underlying disease activity. Although evaluation of anecdotal case reports of response to treatment is hampered by spontaneous fluctuations in the clinical expression of amyloidosis [273], such spontaneous and complete resolution of nephrotic syndrome rarely occurs [274]. These observations strongly suggest a beneficial effect of colchicine in AA amyloidosis and stress the need for controlled trials.

The beneficial effect of colchicine therapy in preventing febrile attacks [275-277] and AA amyloidosis [220, 274, 278] in FMF patients is unequivocal. This effect is dose dependent and if given before there is renal disease, may prevent the development of proteinuria and complications with AA amyloidosis [220, 274, 278]. Colchicine may also be effective in inducing clinical remission of the disease in patients who have proteinuria and even nephrotic syndrome but well preserved renal function [219, 241, 279-283].

Experimental evidence that vitamin C dietary supplements in mice with experimentally induced AA amyloidosis produces resolution of amyloid [284] have not been confirmed [285]. However, amyloidotic mice receiving vitamin C supplements benefited from a highly significant prolongation of survival compared to untreated controls [286]. The mechanism is unknown but these results suggest that oral vitamin C supplements of 200 mg/day which is safe and inexpensive may be useful in the treatment of AA amyloid.

In some cases, the pathology and stimulus for the acute phase response is clearly localised to one site and in such instances, surgical excision of such a localised source of inflammatory stimuli usually lead to a complete and gratifying resolution of clinical symptoms, followed by substantial regression of amyloid deposits. This has been

achieved by tumour resection in renal carcinoma [287], hepatic adenomas [288] and Castleman's disease [289], bowel resection in ulcerative colitis and Crohn's disease [267, 290] and amputations of affected limbs in chronic osteomyelitis [291] that have failed to respond to antibiotics.

Table 8.5 Clinical treatment trials for AA amyloid

Ref	Year	Number of patients	Underlying disease	Treatment Regimes	Duration	5 year survival	Comment
Sch [260]	1977	51	JCA	Ch (continuous) Ch (intermittent) No cytotoxic	Minimum, >12 months	100% ~88% ~70%	Prospective study with Ch dosage 2 mg to 6 mg/day. Survival significantly longer only in patients treated continuously with Ch. Number of patients and characteristics of each group not fully identified. Proteinuria was present in all patients at diagnosis, but in only 36% after > 3 years of continuous Ch treatment.
Falck [266]	1980	60	JRA, AS, JCA	Cy (n=7) No Rx (n=53)	0.5-4.5 years	~ 83% ~ 35%	Prospective study. None of the Cy patients had renal impairment or hypertension, but all had proteinuria.
Berg [261]	1987	14	RA, AS, JCA	Cy or Ch	Median, 12 months	93% (vs 50% for untreated HC)	Prospective study but survival compared with untreated historical controls (HC) with rheumatic diseases and AA amyloidosis. Ch better tolerated than Cy. Dosage adjusted to have peripheral lymphocyte count $< 1.0 \times 10^9$/L.
Ahlm [262]	1987	22	RA	Rx*(n=11) No Rx (n=11)	Mean, 42 months	89% 27%	Only prospective and controlled study so far with both groups matched for renal function, age and interval between diagnosis of rheumatic disease and amyloidosis (mean 16.2 years). *Mostly with podophyllotoxin derivatives, others with Ch, Cy or Azathioprin. Rate of decline of renal function slowed down in Rx group. ESRF developed in 2 patients in Rx gp (1 died, 1 dialysed) and 7 patients in no Rx gp (5 died, 2 dialysed).
David [263]	1993	72	JCA	Ch (n=57) No cytotoxic (n=19)	Mean, 6.5 years	80%** 23.5%**	Retrospective study. Mean interval between JCA amyloidosis is 9.1 years. **Survival figures for 10 years, and after 15 years, 68% of Ch group alive vs no survivors in no Rx group. However, more renal morbidity and mortality no Rx group. Ch dose 0.07-0.12 mg/kg/day, mean cumulative dose 5.03 gm.
Berg [264]	1993	16	RA, JCA, AS	Cy or Ch (n=16)	Median 13 months*	94%**	Prospective uncontrolled study. *Median duration per treatment course and mean number of courses per patient was 2.1. Treatment initiated at signs of disease activity or deterioration in renal function/increased proteinuria. **Refers to renal survival which was 75% at 10 years.

JCA, juvenile chronic arthritis; RA, rheumatoid arthritis; AS, ankylosing spondylitis; Ch, chlorambucil; Cy, cyclophosphamide; HC, historical controls; ESRF, end stage renal failure.

Dialysis-related (β2-M) amyloid

β2-M, the precursor protein of DRA is a LMW protein that forms part of the MHC Class I antigen complex expressed by all nucleated cells. Daily production is estimated at ~ 3 mg/kg [292], almost all of which are filtered and metabolised by the kidneys. It therefore accumulates in renal failure with levels rising 30 to 50 fold in patients on dialysis [156, 293-297]. Although the pathogenesis is unknown, cumulative exposure to high levels of β2-M is clearly an important factor, as β2-M associated systemic amyloidosis have not been described in patients with normal β2-M levels. The biological properties of β2-M as a growth factor capable of remodelling bone [171, 298, 299], its ability to stimulate release of fibroblast collagenase [300] and its affinity for collagen [172] with which it binds in a dose-dependent manner may be important. The process of dialysis which may itself contribute to the daily synthesis of β2-M [295, 301, 302] and may be important in determining clinical expression of the disease [65, 165] is not essential as DRA have been described in a patient with CRF but not yet on dialysis [303].

Prevalence of DRA increases with increasing duration of dialysis and affects virtually everyone who have been dialysed > 20 years [65, 176]. Most patients with DRA present with carpal tunnel syndrome followed by arthralgias of the large and medium sized joints [294]. Joint pains may be symptomatically treated with NSAID but long term efficacy is poor [304]. In contrast to AA amyloidosis, disappointing results have been obtained with the use of colchicine in isolated cases [305]. Low dose oral steroids have given promising results [166] although this will have to be balanced against the potential side effects on an already immuno-compromised dialysis population. The process of HD itself may be a determinant of disease symptomatology [65, 165] but it remains to be seen if conversion from HD to CAPD in patients with DRA may be accompanied by an improvement in symptoms although progression of DRA following conversion to CAPD have already been documented in one such patient [306].

Symptoms associated with carpal tunnel syndrome can be transiently relieved by local steroid infiltration or more definitively by surgical release of the median nerve, which may have to be repeated and cannot be delayed because of the irreversibility of the neurological lesion. Successful relief of shoulder pain after endoscopic resection of the coracoacromial ligament [307] could constitute a major breakthrough in the symptomatic treatment of dialysis arthropathy as the shoulders are one of the most frequently affected joints in this disease. Prosthetic replacement, especially of the hips and knees, may be necessary in patients with pathological fractures as they are unlikely to heal. Destructive spondyloarthropathy, another joint disease frequently associated with β2-M amyloidosis, may lead to severe spondylisthesis with the risk of spinal cord or nerve root compression. Orthopaedic consolidation including osteosynthetic grafts may be a lifesaving procedure in such cases [304].

Specific treatment of DRA as with other types of amyloidosis is aimed at reduction of precursor protein levels which, however, unlike other types of amyloidosis cannot be safely achieved by suppression of synthesis of β2-M. At least 75% of the daily production of β2-M comes from normal cell turnover [65] and suppression of synthesis cannot therefore be achieved without affecting normal cellular function especially of the immune system, the function of which is already undesirably compromised in patients with CRF. Treatment aimed at reduction of β2-M levels is therefore directed towards modifications of dialysis modalities to improve clearance of β2-M, a tubular function of normal kidneys which until recently has been poorly replaced by dialysis. Another goal sought in treatment is the improvement of biocompatibility of membranes as there is *in vitro* evidence that cellulose but not the newer synthetic membranes may stimulate synthesis of β2-M [302, 308] although this has not been confirmed in other studies [309, 310]. However, blood-membrane interaction is not the only source of bioincompatibility because backflow of acetate and endotoxin from dialysis fluid may also contribute to the inflammatory reaction [65, 304, 311, 312], a problem that may be avoided by haemofiltration or reduced by use of ultrapure dialysis fluid [313].

Standard cellulosic membranes used in conventional HD is impermeable to β2-M and bioincompatible. Subsequent development of synthetic membranes such as polyacrylonitrile AN69, PMMA and polysulfone used in high flux dialysis are more biocompatible and more permeable, capable of clearing β2-M by a combination of adsorption (especially with PMMA [314] and AN69 [315] membranes), diffusion transfer, but predominantly, convective transport especially with polysulfone membranes and particularly if used in haemofiltration [316]. Although these dialysis modalities are able to reduce but not normalise β2-M levels and DRA symptomatology does not correlate with β2-M levels [317], a reduction of total body β2-M load could conceivably delay development of the disease in the individual patient. In fact the beneficial effect of high flux dialysis on the development of DRA have been demonstrated but only if such dialysis was employed exclusively or for at least 90% of the total dialysis time [161]. The reasons for this have not been determined but are likely to involve lower β2-M levels and differences in biocompatibility of the different membranes. Prevalence of the disease is significantly reduced in all [161, 173, 174, 318] but one [195] of the studies conducted so far. In patients who already have the disease, high flux dialysis may lead to symptomatic improvement [175, 176] although whether this is accompanied by slowing down of the progression of disease remains to be confirmed. Prevalence of the disease may also be reduced by use of ultrapure dialysis fluid [313] and the theoretical superiority of haemofiltration have not been assessed in trials because of the few numbers of patients on long term haemofiltration.

Intensive removal of β2-M by the combination of high flux dialysis and a haemoperfusion column containing adsorbents with hydrophobic ligands specific for β2-M may be accompanied by improvement in symptoms and even radiological evidence for regression of bone cysts in one case [157]. Confirmation with larger controlled studies are needed but this treatment is likely to prove expensive and with β2-M clearance ~ 300 mg per session is no more efficient than haemofiltration.

Peritoneal dialysis, despite the perfect biocompatibility of the peritoneal membrane that is also highly permeable for small proteins does not protect patients against the risk of DRA [186-188, 319, 320] although disease prevalence may be lower compared to HD populations [173]. It is now one of the most common modes of dialysis and is also capable of clearing β2-M, but at a rate that is dependent on the serum concentration [155]. However, recurrent peritonitis, an important and common complication of CAPD may itself result in AA amyloidosis [321].

Renal transplantation is the most effective method of reducing β2-M levels which fall rapidly to normal limits following a successful transplant [65, 165, 294, 304] and if performed sufficiently early may prevent the development of this disease. In patients with DRA, renal transplantation is effective in providing rapid symptomatic relief of dialysis arthropathy [65, 165, 294, 304] although this is could at least in the short term, be due to a combination of the anti-inflammatory effects of steroids and a consequence of stopping HD [65, 165]. Symptomatic relief achieved with steroids have already been demonstrated in one study [166] and also suggested in 2 patients primed with intravenous steroids for transplantation but not grafted [167]. Prospective evaluation with ^{123}I-SAP scans following transplantation have provided scintigraphic evidence that progression of disease in these patients is halted and may even regress in some cases [165]. This finding is supported by a radiological survey [159] which demonstrated that in contrast to the pre-transplant period, amyloid bone cysts ceased to grow following a successful renal transplant, but neither do they regress although this could simply be a reflection of the failure of bone to heal.

As more patients are being put on dialysis and survive longer, DRA is now a major cause of morbidity in dialysis populations. Early renal transplantation is essential in preventing this otherwise inevitable complication of dialysis, and in patients who are unsuitable for or are unlikely to have a renal transplant but also most at risk of developing DRA such as the elderly [161, 195, 322, 323], alternative dialysis strategies aimed at reduction of β2-M load should be employed early so as to delay the onset of the development of this crippling and potentially fatal complication of dialysis that may in fact limit the duration of long term dialysis.

Hereditary (TTR) amyloid

FAP, an autosomal dominant disorder, is the commonest hereditary form of systemic amyloidosis [17, 40]. Patients characteristically present with mixed sensorimotor peripheral neuropathy that is relentlessly progressive and frequently involve the autonomic nervous system resulting in impaired bladder and bowel function. Crippling disability from neurological disease, weight loss, malnutrition and cardiomyopathy dominate the clinical picture in the latter stages of the disease, resulting in death usually within 10 years of presentation [324]. In all but one [18] of the families studied so far, the disease have been due to point mutations in the transthyretin gene, of which more than 40 mutations have been described.

Until recently, treatment of FAP was limited to supportive measures. Parenteral nutrition may be critical especially in malnourished patients susceptible to pressure sores and infection because of immobility and bladder dysfunction. Unlike other types of systemic amyloidosis, renal involvement is not prominent but may be severe enough to require dialysis. The heart is the main visceral organ that is frequently and fatally involved and indeed in some forms of FAP, cardiomyopathy may be the sole or predominant feature of the disease [113, 149]. Sudden death due to conduction defects may be prevented by prophylactic pacemakers and heart failure due to restrictive cardiomyopathy should be treated although care must be taken to avoid vasodilators. As with most types of systemic amyloidosis, DMSO have been tried but without much success.

Transthyretin is involved in the transport of Vitamin A and thyroid hormones, functions that do not appear to be affected by the mutations. More than 90% [116] of circulating TTR comes from the liver which suggests that replacement of the otherwise functionally normal liver should correct the metabolic defect as have been described for familial hypercholesterolaemia [325] and primary hyperoxaluria [326]. However, amyloid in FAP are derived from both variant and normal TTR [40]. It could not, therefore be assumed that amyloidosis would be halted.

The first liver transplant that was performed for FAP was reported in 1991 [116] and was successful as anticipated in replacing variant TTR with normal TTR in the plasma. At follow up 2 years later [50], disease progression was halted in all 4 patients transplanted. Symptomatic and clinical improvement in 3 of the patients were accompanied by scintigraphic evidence for disease regression in 2 patients who were scanned in contrast to progression of amyloid load and neuropathy in 1 patient who was not transplanted. Successful liver transplantation for FAP have since been reported by other centres [327-329]. Patient and graft survival are similar to those after transplantation for non-malignant liver disorders and there may in fact be less perioperative morbidity with shorter operative time, less blood use and significantly shorter stay in intensive care compared with transplantation for patients with chronic liver failure [327].

Cardiac involvement which may be clinically silent will necessitate prophylactic pacing, at least, perioperatively. In one patient with terminal heart failure, double transplant of heart and liver, the first time this was performed for FAP was undertaken in July 1992 (unpublished observations). Two years after his double transplant, serial autonomic function and nerve conduction studies demonstrated progressive improvement, providing unique evidence for regression of neuropathy that have not previously been demonstrated in other patients despite the halt in disease progression.

The successful treatment, at least in the short term, of FAP by liver transplantation should be confirmed in longer term studies. In addition, patient selection and timing of the operation must be established. Patient selection is complicated by variations in disease expression; TTR mutation are not always penetrant, and even within the same family phenotypic expression may vary considerably [330]. Individuals with late-onset and slowly progressive disease are least likely to benefit as are those who are already severely affected and likely to suffer perioperative morbidity or mortality. Timing is therefore crucial for liver transplantation to be optimally effective. Individuals known to have the mutation must be monitored closely for neurological and cardiac involvement. Clinical evidence for disease must be confirmed histologically so that early liver transplantation can be considered especially in a young patient with rapid disease

progression. Genetic counselling is mandatory and of particular significance in younger members of the family who are at risk of having inherited and therefore transmitting the mutation. Gene therapy which will have to be aimed at selective elimination of expression of variant TTR is unlikely to be developed before conditions where therapy is aimed at replacing a deficient but not defective product.

In summary, liver transplantation offers the only effective treatment for FAP. Disease progression is halted and may even be reversed. Patient selection and timing needs to be established and optimised to provide maximal benefit especially in areas where the disease is endemic and organ demand will considerably exceed organ supply. Finally, liver transplantation may have a role in the treatment of hereditary amyloidosis due to variant apolipoprotein AI which is partly produced by the liver and partly by the small intestine. Reduction in supply of precursor variant apoAI following a liver transplant may slow down progression of the disease in which the amyloid consists exclusively of fragments of variant apoAI [19].

Localised amyloid

Localised amyloid may affect virtually any organ systems. It occurs in the absence of primary disease of the affected organ and also in the absence of systemic diseases. The fibril precursor protein of localised amyloid deposits are usually derived from locally produced proteins or from immunoglobulin products of a localised clone of plasma cells (Table 1.1). Clinically significant deposits result in impaired organ function or more commonly, cause mechanical problems such as hoarseness in laryngeal amyloid, obstruction or haematuria in urinary tract amyloid, restricted eye movements and proptosis in orbital amyloid (see Chapter 7). More importantly, localised amyloid often resembles a tumour and is misdiagnosed as a malignancy leading to unnecessary surgery such as ureteronephrectomy [331, 332]. A high index of suspicion and good quality histology including Congo red staining is therefore critical for an accurate diagnosis.

Treatment of localised amyloid is largely symptomatic and usually achieved by local excision. Recurrence necessitating repeat surgery may occur and long term follow up is therefore recommended. With laryngeal amyloid, local endoscopic resection is usually successful [333] although reconstructive surgery may be

necessary [334]. Laser therapy [335] may have a role especially with large deposits in the trachea or bronchi causing airways obstruction.

The most common procedure described for treatment of amyloid of the upper urinary tract was resection and reanastomosis of the ureter [332]. Successful treatment with renal autotransplantation [336], ileal conduit surgery and ileal ureter [332] have also been reported. Ureteronephrectomy is usually unnecessary and almost always performed because of a diagnosis of transitional cell carcinoma which has similar radiographic findings [331, 332]. Amyloid of the lower urinary tract may be treated with transurethral resection and electrocoagulation [337, 338] whilst extensive lesions may require segmental resection of the urethra and end-to-end anastomosis or perineal urethrotomy [339]. Radical treatment with total cystectomy and urinary diversion may be necessary in extensive amyloid of the bladder [340].

Primary localised cutaneous amyloid often causes hyperpigmentation and pruritus which may follow a chronic intractable course. There have been isolated reports of the beneficial effect of therapy with the aromatic retinoid, etretinate, dermabrasion, topical DMSO [341], ultraviolet B (UVB) [342] and even carbon dioxide laser therapy [343]. Other reports however, described poor results with strong topical corticosteroids and keratolytic agents such as salicylic acid ointment [344].

Role of dimethyl sulphoxide

DMSO is a highly hygroscopic solvent with minimal toxicity. It has an anti-inflammatory action and may reversibly block the thermoprecipitation reaction of Bence-Jones proteins and dissolve amyloid fibrils *in vitro* [345]. Reports that DMSO may be used advantageously in AA [346], AL [205] and hereditary systemic amyloidosis [347] have yet to be confirmed. A considerable social disadvantage of the drug which is also likely to limit treatment compliance is the odour it produces on the breath because a small percentage of the drug is excreted as dimethysulphide. It is unlikely that DMSO will have a significant place in the modern treatment of amyloidosis.

Role of colchicine

Colchicine is an antimitotic agent that works by interfering with cell division. Its effectiveness as an anti-inflammatory agent in suppressing acute attacks of FMF was first demonstrated in 1972 [277]. Since then colchicine have been used in the treatment of AA [220, 267-272, 274, 278] AL [223, 225, 241-247] and even β2-M [305] amyloidosis. However, its beneficial effect have so far been demonstrated unequivocally only in the prevention of amyloidosis of FMF [220, 274, 278]. This effect is dose dependent and achieved only with a daily dosage of at least 1.5 mg. Interestingly, this protective effect of colchicine is seen even if it fails to prevent attacks of FMF [274, 278, 348], supporting the hypothesis that amyloidosis due to FMF is a phenotypically independent trait of the disease [348, 349]. Colchicine also prevents recurrence of amyloidosis in FMF patients who have received a kidney transplant [278]. Since prolonged spontaneous remissions of FMF rarely occurs [278, 350], it is likely that colchicine will have to be continued indefinitely to remain effective. The long term outcome of colchicine treatment remains unknown although colchicine have been given to children [351] and pregnant mothers [352] without adverse effects. In FMF patients who are already affected by amyloidosis, colchicine may be able to reduce proteinuria and even promote resolution of nephrotic syndrome [241, 279-283]. Response may be rapid although it is usually seen after 6 to 12 months of treatment [278].

Isolated but numerous case reports [267-272] suggest that colchicine may also be beneficial in the treatment of AA amyloidosis secondary to other chronic inflammatory conditions, resulting in reduction of proteinuria and even improvement in renal function. These encouraging findings will however need to be confirmed by controlled clinical trials.

The suggestion that colchicine on its own may be useful in the treatment of AL amyloid [245] especially if kidneys are the main organ system affected, and even improve survival have not been confirmed [244, 247]. Furthermore, recent evidence suggests that prolonged survival achieved with melphalan and prednisolone is not improved further by adding colchicine to the regime [247]. Finally, in dialysis-

related amyloid, results of treatment with colchicine have proved disappointing [305].

Role of renal replacement therapy

Renal failure is a major cause of morbidity and mortality in AA, AL and some forms of hereditary systemic amyloidosis. The availability of renal replacement therapy (RRT) with HD from the 1960's and CAPD from the 1980's have considerably improved the prognosis [210-212] in what was otherwise an invariably fatal complication of amyloidosis. Survival of AL patients requiring dialysis compares favourably with those not requiring dialysis [213] and it is likely that the much improved survival reported in AA patients given alkylating cytotoxic treatment [260-264] is partly attributable to replacement of renal function by dialysis. However, the benefits of dialysis is limited by the progressive and systemic nature of the disease. Survival on dialysis is therefore significantly poorer than in patients with renal failure due to nonsystemic diseases but comparable to patients with renal failure due to a multisystem disease such as diabetes [210, 211]. Fistula failure, gastrointestinal complications and especially intradialytic hypotension are problems encountered more frequently in amyloidosis patients [210]. However, other indices of morbidity such as infection rate including peritonitis rates in CAPD patients and number of hospital admissions per patient are similar to that seen in the general dialysis population [210].

Survival in dialysis patients with AL amyloidosis may be shorter than that of patients with AA amyloidosis [213] although in a study involving 48 patients [210] most of whom had AA amyloidosis, median survival on dialysis of 52 months was comparable between the two sub-groups but better than the median survival of 8.2 months of 37 AL patients in another study [213]. Cardiovascular and circulatory complications associated with amyloidosis are the main causes of death for both AA and AL patients [210, 211, 213]. Clinically silent but echocardiographically evident cardiac amyloid is the most important predictor of poor survival in dialysis patients with AL amyloidosis [213] whilst acute deterioration of renal function, short disease duration, age and evidence for extra-renal amyloid predicts poor survival in both

AA and AL amyloid patients [210, 211]. The adverse effect of multisystem involvement is best illustrated by long term survivors, most of whom have no evidence for extra-renal amyloidosis [210, 211, 213].

There have been no prospective studies comparing HD with CAPD which have several theoretical advantages. It is not associated with the circulatory stress of intermittent HD, and haemoglobin levels are better preserved on CAPD. Both these factors may be important in the presence of cardiomyopathy and coronary artery disease, especially since intradialytic hypotension is the commonest complication in these patients. In addition, vascular access required for HD in a vessel potentially infiltrated with amyloid may be a problem, although fistula failure may [210], or may not [211] be more common in amyloidosis. Finally, immunoglobulin light chains which are not cleared by HD may be cleared by CAPD [212, 353] and can therefore potentially slow down progression of AL amyloid. On the other hand, attacks of FMF may curiously be reduced following commencement of HD [354]. In published studies, all retrospective [210, 211, 213], HD was more commonly employed than CAPD and survival for both modalities of dialysis were similar despite a significantly higher proportion of patients with cardiac amyloid in the CAPD group in one study [211]. Such indirect evidence that dialysis survival may be superior in CAPD is supported by the observation that circulatory stress of intermittent HD may contribute to the death of amyloidotic patients with cardiac involvement, an important cause of death in these patients. Despite concerns raised regarding the efficacy of peritoneal membranes potentially infiltrated with amyloid [355], both dialysis and ultrafiltration appears adequate in these patients [211-213]. However, although peritonitis rates may be comparable to the general CAPD population [212, 355], temporary conversion to HD should be considered during chemotherapy for AL amyloid although this may not always be necessary [356].

Amyloidosis continue to progress on dialysis and is responsible for much of the morbidity seen in these patients. Gastrointestinal involvement may cause pseudo obstruction, anorexia or nausea and vomiting [357] which may be readily but wrongly attributed to uraemia. Much of the circulatory failure seen in systemic amyloidosis especially in AA patients could be due to previously unrecognised adrenal dysfunction due to amyloid [358] and a clinically silent but functionally

important hypoadrenal state should therefore be sought by appropriate stimulation with synacthen. Hypotension, in addition, could also be due to autonomic neuropathy or cardiomyopathy even in AA patients. Clinically significant cardiac involvement in AA amyloid may be more common than previously suspected especially when amyloidosis is allowed to progress in patients maintained on dialysis [359] and is suggested by the observation that cardiomyopathy and circulatory failure are also the most common causes of death on dialysis even in AA patients [210, 211]. Finally, most patients die of progressive systemic amyloid disease rather than complications of dialysis, suggesting that survival on dialysis may be improved further if progression of amyloid can be halted.

Role of organ transplantation

Since it was first performed in 1968 [360], renal transplantation have been established as an effective treatment of renal failure due to AA and AL amyloidosis [214-217, 249, 265]. Five year patient and graft survival at 65% and 62% respectively [214] compares favourably with survival in other groups of patients including those with nonsystemic causes of renal failure. In 2 large studies [214, 215], survival was similar between AL and the larger group of AA patients. Age was found to have a major influence, with survival significantly higher in those aged < 45 years [214, 216]. There was evidence that survival may be improved further by early transplantation [215] although this was not confirmed in another study [214] which demonstrated no difference between those transplanted before and those transplanted after dialysis-dependent renal failure although the mean time spent on dialysis was short at 9.6 months.

A striking finding is the very high early post-operative mortality due to infection [214, 215, 217, 249]. The need for anti-rejection therapy in addition to standard immunosuppressive treatment especially during the first 3 months following transplantation is the period in which amyloidotic patients are most vulnerable, with most succumbing with fatal consequences to overwhelming pneumonia and septicaemia. Such critical susceptibility to infection could be due to their already immunocompromised status which may in part be related to splenic

involvement in AL and particularly AA amyloidosis in which it is universally affected [58]. No information was provided as to the organisms involved but morbidity and mortality can clearly be potentially reduced in these patients with appropriate precautions. Prophylactic antibiotics at least in the first 3 post-operative months should be considered together with appropriate isolation to minimise exposure during rejection episodes. Modifications of anti-rejection therapy currently employed may be necessary but the most effective precaution would be to reduce the risk of rejection and therefore the frequently fatal consequences of anti-rejection therapy. Careful selection of patients and matching of donor organs are therefore of paramount importance. The development and use of newer immunosuppressive treatment such as cyclosporine have led to improved graft survival [214, 217] but whether the number of rejection episodes are reduced was not confirmed.

In contrast, outcome improves considerably after 1 year. Mortality is markedly reduced and the number of anti-rejection episodes overall may in fact be lower and graft survival better than that of non-amyloidotic patients [215]. Recurrence of amyloid in the transplanted kidney is an obvious concern, but this affects only up to 26% [361] of patients surviving > 1 year and very rarely causes graft failure even after 15 years of follow-up [214]. This observation contrasts sharply with the relentless progression of amyloidosis seen in dialysis patients. Most patients in the dialysis and transplant studies published so far have AA amyloidosis due to chronic inflammatory diseases and it is likely that post-transplant immunosuppressive treatment have a beneficial effect on the underlying disease process in these patients. Amyloidosis on the other hand may be accelerated in dialysis patients because of the associated acute phase response that may occur in bioincompatibility reactions during HD and peritonitis in CAPD. These observations which will need to be confirmed, provide further evidence that effective management of dialysis and transplant patients must include monitoring and treatment of the underlying disease process, successful control of which can reduce patient morbidity and mortality.

The role of organ transplantation in the treatment of terminal organ damage caused by amyloidosis was recently extended to include heart transplantation [47,

218]. In the only published study so far which involved 10 patients [218] survival was not significantly worse than patients transplanted for other cardiac diseases. This encouraging finding is made all the more remarkable by the fact that all 10 patients had AL amyloid and many had evidence for multisystem disease. In addition, chemotherapy for AL amyloid pre or post-transplant may not have been given although this important information was not provided in the report. All 4 patients known to have died after 50 months of follow-up succumbed to systemic progression of amyloidosis, a process that may be halted by appropriate post-transplant chemotherapy [47]. Cardiac AL amyloidosis carries a poor prognosis with median survival of 6 months [16, 53, 223]. Such patients are therefore unlikely to see the benefits of chemotherapy which have a median response time of 11.7 months [246]. Heart transplantation will therefore not only be life saving and improve the prognosis considerably, but will also allow time for chemotherapy to take effect. Chronic shortage of donor organs [218] should not be used as an excuse to deny the opportunity of life saving heart transplantation in this small but select group of patients in whom survival may be substantially prolonged with effective post-transplant chemotherapy. Young patients with minimal multisystem involvement are most likely to benefit. Patient assessment and selection is therefore important. Pre-operatively, quantification and distribution of total body amyloid must be determined and this at the moment can only be achieved *in vivo* by a radio-labelled SAP scan [58, 59]. Post-operatively, chemotherapy aimed at preventing systemic progression of amyloidosis must be given as this may further improve survival which is already substantially prolonged by the life saving transplant.

Until recently, the role of organ transplantation in amyloidosis was limited to replacement of target organs terminally damaged by the disease. This palliative role of organ transplantation is well established and considerably improves prognosis although the underlying disease process may continue and necessitate specific treatment. Recently, a potentially curative role of organ transplantation have been established in the treatment of hereditary systemic TTR amyloidosis [50, 116]. The liver accounts for > 90% [116] of total body TTR production and replacement of the functionally normal but metabolically defective liver in hereditary systemic amyloidosis due to variant TTR results in replacement of variant TTR from the

plasma, halt in disease progression and may even be accompanied by disease regression [50]. Such a curative role for transplantation have also been attempted by bone marrow transplantation in the treatment of systemic AL amyloidosis in one patient [251]. However, such a radical approach is unlikely, at least in the near future, to replace the combined use of chemotherapy and solid organ transplantation in treatment of AL amyloid.

Future Developments

Treatment aimed at reducing supply of precursor proteins are not always successful or may be too late in patients who already suffer severe target organ damage. However, the demonstration that SAP is a universal constituent of all amyloid deposits, its inherent resistance against digestion in the presence of calcium, and *in vitro* evidence that it protects fibrils against degradation [362] may provide the basis for future treatment directed specifically towards degradation and mobilisation of amyloid. The specific binding of SAP to amyloid fibrils, a property utilised with considerable success in SAP scintigraphy suggests that SAP may also be used as a targeting molecule or 'magic bullet' to deliver pro-inflammatory agents. The mobilisation of macrophages and release of proteinases would therefore be maximal at sites of specific SAP localisation, thereby encouraging local amyloid reabsorption. However, this scenario may already be superseded by the recent identification to atomic resolution of the three dimensional structure of SAP [36]. Availability of the complete structure to 2Å resolution of SAP and determination of its ligand binding sites now offer the opportunity of direct modelling of competitive inhibitors of SAP binding and for producing binding site homologues, either of which could be used as drugs to displace SAP from amyloid deposits *in vivo*. This would open up new avenues for treatment of amyloidosis, enabling the body to mobilise and degrade the fibrils which may otherwise be inappropriately protected by SAP. Finally, the advent of genetic therapy means that expression of precursor proteins especially variant proteins of hereditary systemic amyloidosis may be selectively eliminated but this is unlikely to be developed before conditions where therapy is aimed at replacing a deficient but not defective product.

General Conclusions

The solubility of amyloid fibrils in water but not salt forms the basis of the standard amyloid fibril extraction technique [8], enabling fibrils to be separated from tissue proteins which are soluble in both salt and water. However, such isolation of amyloid fibrils have until recently been possible only from gram quantities of tissue obtained at post-mortem or during an operation. A novel method of "mini-fibril" extraction which for the first time has enabled amyloid fibrils to be isolated and characterised from tiny milligram quantities of biopsy tissue is described [10].

The studies on families with hereditary systemic amyloidosis [111, 113, 130, 363] illustrates the potential application of combined laboratory and clinical investigative techniques in characterising the molecular and genetic basis of the disease, in relation to the clinico-pathological features of the disease. The investigations of these families are entirely dependent initially on the immunohistochemical identification of the precursor protein which then allows the appropriate gene to be identified, extracted and amplified by PCR for DNA sequencing. This routine and most basic of molecular biological investigative techniques was sufficient for identification of these mutations. However, not all mutations are pathogenic or amyloidogenic as clearly illustrated for example by the demonstration of more than 20 electrophoretic (non-amyloidogenic) variants of apoAI following the screening of 32,000 blood samples [86]. Concordance between mutation and disease will therefore have to be established by the demonstration of variant sequence in isolated amyloid fibrils.

In the two families where elaborate characterisation of fibril proteins by amino acid sequencing and electrospray mass spectrometry was made possible by the development of the "mini-fibril" extraction technique, the fibrils were found to be derived exclusively from variant proteins [111, 363]. Such precise characterisation provided novel information on the heterogeneity of the cleavage sites of the precursor proteins with C-terminal raggedness in the amyloidogenic apoAI fragments. These fragments are however, electrophoretically homogeneous in having an extra positive charge despite being derived from different apoAI

variants. This suggests that the acquisition of an extra positive charge in the *N*-terminal amyloidogenic fragments of apoAI rather than substitution by any specific residue is the key amyloidogenic event. It is possible that the extra positive charge mediates a pro-amyloidogenic destabilisation of protein structure, promoting fibrillogenic aggregation with proteolytic cleavage as a secondary phenomenon, or they may facilitate proteolytic cleavage of the native molecule to yield fibrillogenic fragments. With the hereditary transthyretin amyloidoses, the inherent amyloidogenic potential of wild-type transthyretin was increased for the variants, resulting in disease of a different phenotype. These inherited amyloidogenic variants therefore provide valuable models for analysing the pathogenesis of amyloidosis, and in addition, their identification allows genetic counselling and informed testing to be conducted in the affected families.

Phenotype in these families were extensively studied clinically, biochemically, histologically and scintigraphically, providing unique information on the pattern of distribution of amyloid with invariable involvement of the kidneys in families with hereditary renal amyloidosis. A prodromal phase of sub-clinical amyloid deposition together with poor correlation between amyloid load and organ dysfunction was demonstrated. The observation that the natural history of the disease may vary even amongst families with the same mutation, suggests that factors other than the mutation are important in determining clinical expression of the disease. The autosomal dominant pattern of inheritance, concordance between mutation and disease, and the virtually complete penetrance of these mutations were also demonstrated.

Studies on acquired forms of amyloidosis were focused on the effect of renal transplantation [191] and CAPD [177] on DRA, an invariable complication of long term dialysis. In addition to confirming the rapid resolution of symptoms of DRA following a successful transplant, the long term preservation of such symptomatic improvement despite withdrawal of steroids were reported for the first time. Unique radiological and scintigraphic regression of DRA deposits post-transplant were also demonstrated, in contrast to progression of the disease in patients maintained on haemodialysis. Amongst long term CAPD patients, scintigraphic evidence for the disease appears to be comparable to histological evidence for the

disease in HD patients [183]. However, CAPD patients were less symptomatic, suggesting that clinical expression of the disease may be modulated by dialysis modality.

In both HD and CAPD patients, CTS was the commonest presenting feature of DRA and scintigraphically, the wrists were always affected in patients with a positive scan. Age and duration of dialysis were confirmed as the most important risk factors for the development of DRA. Histological and scintigraphic studies demonstrated that in both the hereditary and acquired forms of amyloidosis, there was an asymptomatic prodromal phase, which may last for several years, and in the case of DRA, the disease was frequently underestimated clinically and radiologically.

Finally, the first case of primary localised orbital amyloidosis [10] was fully characterised and the CH3 domain of γ heavy chains was identified as the precursor protein. This is the third case of heavy chain-related (AH) amyloid but the first case of immunoglobulin-related (both AL and AH) amyloid where the fibril precursor protein is derived exclusively from a constant domain with apparently no contribution from the variable domains. The inherent amyloidogenic potential of heavy chains demonstrated by these three cases of heavy chain amyloid, two of whom had phenotype similar to AL amyloidosis [203, 204], suggests that some cases of monoclonal immunoglobulin-related amyloid (usually clinically designated as AL amyloid), may be AH amyloid derived from heavy chain components of these monoclonal proteins.

Appendix I - Thesis Work Presented At Meetings

Parts of the thesis was presented as papers at the following meetings:

1. **Tan SY**, Madhoo S, Brown E, Gower P, Irish A, Winearls C, Clutterbuck EJ, Pepys MB, Hawkins PN. Effect of renal transplantation on dialysis-related amyloid deposits: Prospective evaluation by ^{123}I-SAP scintigraphy. *XXXIst Congress of the European Dialysis and Transplant Association European Renal Association*, Vienna, Austria, 3-6 July 1994.

(Nephrol. Dial. Transplant. 1994:9;1016)

2. **Tan SY**, Irish A, Winearls C, Brown E, Gower P, Clutterbuck EJ, Madhoo S, Lavender JP, Pepys MB, Hawkins PN. Effect of renal transplantation on dialysis-related amyloid: A prospective 5 year study. *The Renal Association (UK) Autumn Meeting,* University College, London; 17-18 October 1994.

(Nephrol. Dial. Transplant. 1995:10;719)

3. **Tan SY**, Irish A, Winearls C, Brown E, Gower P, Clutterbuck EJ, Madhoo S, Lavender JP, Pepys MB, Hawkins PN. Long term outcome of renal transplantation on dialysis-related amyloid deposits and disease symptomatology: A prospective 5 year study. *The American Society of Nephrology 27th Annual Meeting,* Orlando, Florida, 26-29 October 1994.

(J. Am. Soc. Nephrol. 1994:5;479)

4. **Tan SY,** Booth DR, Booth SE, Campistol JM, Bruguera M, Caballeria J, Hutton T, Hsuan JJ, Totty NF, Nguyen O, Hutchinson WS, Hawkins PN, Pepys

MB. Hereditary systemic amyloidosis caused by a unique apolipoprotein AI deletion mutation. *The Renal Association (UK) Spring Meeting,* Newcastle upon Tyne, 11-12 April 1995.

(Nephrol. Dial. Transplant. 1995:10;2394-2395)

5. Booth DR, **Tan SY (Presenting Author),** Booth SE, Hsuan JJ, Totty NF, Nguyen O, Tennent GA, Hutchinson WS, Vigushin DM, Thomson N, Soutar AK, Hawkins PN, Pepys MB. Hereditary renal amyloidosis caused by a new apolipoprotein AI variant, Trp50Arg. *XXXIInd Congress of the European Dialysis and Transplant Association European Renal Association*, Athens, Greece, 11-14 June 1995.

(Nephrol. Dial. Transplant. 1995:10;943)

6. **Tan SY,** Booth DR, Booth SE, Campistol JM, Bruguera M, Caballeria J, Hutton T, Hsuan JJ, Totty NF, Nguyen O, Hutchinson WS, Hawkins PN, Pepys MB. Hereditary systemic amyloidosis caused by a unique apolipoprotein AI deletion mutation. *XIIIth International Congress of Nephrology,* Madrid, Spain, 2-6 July 1995.

(Kidney Int. (in press))

7. **Tan SY,** Booth DR, Debusmann ER, Pepys MB, Hawkins PN. Characterisation and therapy of hereditary renal amyloidosis in a German family. *The Renal Association (UK) Autumn Meeting,* University College Hospital, London, 9-10 October 1995.

(Nephrol. Dial. Transplant. (in press))

8. Pepys MB, Booth DR, Booth SE, **Tan SY,** Persey MR, Hutchinson WL, Campistol JM, van Zyl-Smit R, Hawkins PN. New variants of apoAI. The first deletion mutations causing hereditary amyloidosis. *90th Annual Meeting of The Association of Physicians of Great Britain and Ireland,* Manchester 11 & 12 April 1996.

Parts of the thesis work was presented as abstracts at the following meetings:

1. Hutchinson WL, Mather SJ, Staltieri M, Noble GE, Vigushin DM, **Tan SY**, Seymour A, Pepys MB, Hawkins PN. Scintigraphic imaging of amyloid deposits with ^{99m}Tc-labelled serum amyloid P component. *The VIIth International Symposium on Amyloidosis,* 11-15 July 1993, Kingston, Ontario, Canada.

(In: Kisilevsky R, Benson MD, Frangione B, Gauldie J, Muckle TJ, Young ID, eds. *Amyloid and Amyloidosis 1993.* Park Ridge, New Jersey: The Parthenon Publishing Group; 1994:682-684).

2. **Tan SY**, Madhoo S, Brown E, Clutterbuck EJ, Lavender JP, Pepys MB, Hawkins PN. Continuous ambulatory peritoneal dialysis and dialysis-related amyloid: Clinical, radiological and ^{123}I-SAP scan findings in 7 patients. *The Renal Association (UK) Autumn Meeting,* University College, London; 17-18 October 1994.

(Nephrol. Dial. Transplant. 1995:10;741-742)

3. **Tan SY**, Madhoo S, Brown E, Clutterbuck EJ, Lavender JP, Pepys MB, Hawkins PN. Continuous ambulatory peritoneal dialysis and dialysis-related amyloid: Clinical, radiological and ^{123}I-SAP scan findings in 7 patients. *The American Society of Nephrology 27th Annual Meeting,* Orlando, Florida, 26-29 October 1994.

(J. Am. Soc. Nephrol. 1994:5;479)

4. Booth DR, Booth SE, Persey MR, **Tan SY,** Madhoo S, Pepys MB, Hawkins PN 3 new amyloidogenic TTR mutations: Pro12, Glu18, and Val33 *The 3rd International Symposium on Familial Amyloidotic Polyneuropathy and other Transthyretin Related Disorders,* Lisbon, Portugal, 27-29 October 1995.

5. **Tan SY,** Booth DR, Debusmann ER, Pepys MB, Hawkins PN Characterisation and therapy of hereditary renal amyloidosis in a German family. *The American Society of Nephrology 28th Annual Meeting,* San Diego, California, 5-8 November 1995.

(J. Am. Soc. Nephrol. 1995:6;728)

Appendix II - Publications Arising From Thesis Work

1. **Tan SY**, Pepys MB. Amyloidosis. *Histopathology.* 1994; 25:403-414.

2. **Tan SY**, Murdoch IE, Sullivan TJ, Wright JE, Truong O, Hsuan JJ, Hawkins PN, Pepys MB. Primary localised orbital amyloidosis composed of the immunoglobulin γ heavy chain CH3 domain. *Clinical Science.* 1994;87:487-491.

3. **Tan SY.** Amyloidosis. *Continuing Medical Education (CME) Review.* 1994: 4; 61-70.

4. Booth DR, **Tan SY**, Hawkins PN, Pepys MB, Frustaci A. A novel variant of transthyretin, 59Thr→Lys, associated with autosomal dominant cardiac amyloidosis in an Italian family. *Circulation.* 1995;91:962-967.

5. **Tan SY**, Pepys MB, Hawkins PN. Treatment of Amyloidosis: An In-Depth Review *American Journal of Kidney Diseases.* 1995:26;267-285.

6. Booth DR, **Tan SY,** Booth SE, Hsuan JJ, Totty NF, Nguyen O, Hutton T, Hutchinson WL, Thomson N, Soutar AK, Hawkins PN, Pepys MB. A new apolipoprotein AI variant, Trp50Arg, causes hereditary amyloidosis. *Quarterly Journal of Medicine*. 1995:88;695-702.

7. Hawkins PN, **Tan SY**, Pepys MB. General aspects of amyloidosis In: *Oxford Monographs on Clinical Nephrology: Dialysis Amyloidosis* (van Ypersele C, Drueke T, eds.), Oxford University Press, Oxford 1996; 34-68.

8. **Tan SY**, Irish A, Winearls C, Brown E, Gower P, Clutterbuck EJ, Madhoo S, Lavender JP, Pepys MB, Hawkins PN. Long term outcome of dialysis-related amyloid deposits and disease symptomatology in patients undergoing renal transplantation: A prospective 5 year study. *Kidney International* 1996 (in press).

9. Hawkins PN, **Tan SY,** Pepys MB. The patient with amyloidosis. In: *Oxford Textbook of Clinical Nephrology, 2nd Edition* (Davison, AM, Cameron, JS, Grunfeld, JP, Kerr, DNS, Ritz, E, eds.), Oxford University Press, Oxford, (in press).

10. **Tan SY,** Booth DR, Booth SE, Tennent GA, Hutchinson WL, Hsuan JJ, Totty NF, Nguyen O, Soutar AK, Hawkins PN, Bruguera M, Caballeria J, Sole M, Campistol JM, Pepys MB. Hereditary hepatic and systemic amyloidosis caused by a new deletion/insertion mutation in the apolipoprotein AI gene. *Journal of Clinical Investigation* 1996 (in press).

11. Murdoch, IE, Sullivan TJ, Moseley I, Hawkins PN, Pepys PN, **Tan SY,** Garner A, Wright JE. Primary localised amyloidosis of the orbit. *British Journal of Opthalmology* 1996 (in press).

12. Pepys MB, Tennent GA, Booth DR, Bellotti V, Lovat LB, **Tan SY,** Persey MR, Hutchinson WL, Booth SE, Madhoo S, Soutar AK, Hawkins PN, Van Zyl-Smit R, Campistol JM, Fraser PE, Radford SE, Robinson CV, Sunde M, Serpell LC, Blake CCF. Molecular mechanisms of fibrillogenesis and the protective role of amyloid P component: two possible avenues for therapy. In *The nature and origin of amyloid fibrils.* Wiley, Chichester, Ciba Foundation Symposium 1996; 73-89.

13. **Tan SY,** Hawkins PN, Pepys MB. The hereditary renal amyloidoses: Report of a family and review of the literature. *(submitted to Kidney International).*

14. **Tan SY**, Baillod R, Brown E, Farrington K, Soper C, Clutterbuck EJ, Percy M, Madhoo S, Pepys MB, Hawkins PN. Continuous ambulatory peritoneal dialysis and dialysis-related amyloid: A clinical, radiological and scintigraphic study. *(submitted to Nephrology Dialysis Transplantation 1996).*

15. Familial amyloid polyneuropathy due to a novel transthyretin mutation in a Colombian family. (**Tan SY,** et. al., *for submission to Human Genetics*).

16. Hereditary renal amyloidosis associated with a mutant fibrinogen α-chain. (Hawkins PN et. al., *for submission to Kidney International*).

Appendix III - Proposals For Future Research Work

Characterisation of amyloid proteins

Primary cutaneous (lichen) [364] and intra-tumour amyloidosis [27] are rarely seen in the West, but common in South America and the Far East. The precursor proteins for these well recognised forms of amyloidosis have not yet been identified and attempts will therefore be directed towards the characterisation of these precursor proteins, suspected to be keratin [365] in primary cutaneous amyloidosis. However, the deposits in intra-tumour amyloid do not react immunohistochemically with antibodies directed against known amyloid proteins [27], suggesting that the fibrils may be derived from a novel amyloidogenic protein. Amyloid fibrils will therefore be isolated and purified as previously described [10], and analysed by Congo red staining, electron microscopy, SDS-PAGE, and immunoblotting. Precise characterisation of fibril protein by amino acid sequencing and determination of molecular masses of extracted fibrils by electrospray mass spectrometry will be performed in collaboration with Dr Justin Hsuan's laboratory at the Ludwig Institute for Cancer Research, London, and with Therese Hutton of Fisons VG Biotech, Altrincham, Cheshire. Scintigraphic studies of affected cases by ^{123}I-SAP scans, an *in vivo* technique for the diagnosis of systemic amyloidosis [58, 59], will be conducted in collaboration with Professor M B Pepys and Dr P N Hawkins of The Royal Postgraduate Medical School, London.

In addition to contributing to the understanding of the pathogenesis of amyloidosis, the identification of the precursor protein of intra-tumour amyloid which is classically associated with nasopharyngeal carcinoma (NPC) [14, 27], has potentially important clinical applications. This precursor protein may be used as a marker for NPC, the commonest gastrointestinal malignancy to affect the Oriental population, and may therefore be used as a screening test for the tumour and for following clinical progress following treatment. With primary cutaneous amyloidosis, the characterisation of the precursor protein will be followed by genetic studies to identify the mutation(s) involved

in hereditary forms of the disease which has an autosomal dominant pattern of inheritance [366].

Role of advanced glycation end-products (AGE) in amyloidogenesis

The role of AGE modified forms of precursor proteins have been established in the deposition of β2-M amyloid of dialysis arthropathy [367] and β-amyloid of Alzheimer's Disease [368]. The role of AGE modified precursor proteins in AA, AL, transthyretin, primary cutaneous and intra-tumour amyloid will therefore be studied by immunohistochemical staining of paraffin-fixed biopsies by the peroxidase-anti-peroxidase method, using mouse monoclonal anti-AGE antibodies [369] to be supplied by Dr Toshimitsu Niwa of Nagoya University Branch Hospital, Japan. Immunohistochemical evidence for the presence of AGE modified proteins will then be confirmed by immunoblotting of extracted fibrils using the same monoclonal antibodies.

Role of advanced glycation end-products (AGE) in the pathogenesis of ultrafiltration failure in peritoneal dialysis

It has been suggested that diabetics may have poorer ultrafiltration on CAPD due to increased capillary permeability caused by diabetic microvascular disease, a process mediated at least in part, by advanced glycosylation [370, 371]. Similarly, prolonged exposure of peritoneal membrane to concentrated glucose solutions used in peritoneal dialysis may eventually lead to ultrafiltration failure via a similar mechanism which involves AGE modification of structural membrane proteins [372]. The peritoneal

membrane transport characteristics of diabetics with renal failure will therefore be studied using the peritoneal equilibration test (PET) [373], and compared with patients with renal failure due to systemic amyloidosis, a disease which may also affect peritoneal membrane structure and function. The control group will consist of patients with renal failure due to non-systemic diseases such as the primary glomerulonephritides. Correlation between ultrafiltration characteristics and presence of AGE modified proteins in the circulation and peritoneal membrane will be established. AGE-modified haemoglobin [374] and plasma pentosidine [375] will be used as circulating markers of advanced glycosylation whilst tissue glycosylation will be determined by quantifying accumulation of peritoneal membrane pentosidine [375] using biopsies taken at the time of catheter insertion, renal transplantation or at post-mortem. This cross-sectional study will be followed by a longitudinal study to determine the changes, if any, in peritoneal membrane structure and transport characteristics in all 3 groups of patients.

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