Advances in the Diagnosis and Treatment of Amyloidosis and Related Disorders

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Declaration

I, Rabya Hussain Sayed, confirm that the work presented in this thesis is my own.

I have declared where information has been derived from other sources.

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Abstract

Background

Amyloidosis is a disorder arising from the variable physiological effects of dysregulated, extracellular protein deposition. There are >30 different subtypes, all possessing the same histological characteristics and the two major organs affected are the kidneys and the heart.

Aims and Hypothesis

- To evaluate current UK histological practices leading to a misdiagnosis of amyloidosis
- To establish proteomics as a new diagnostic technique for identifying amyloid, in the UK
- To investigate the usefulness of a relatively new biomarker i.e. Retinol Binding Protein (RBP), across amyloid subtypes and correlate values with biopsy findings, which has not previously been done
- To identify the cause of death in patients with Stage III/ IV cardiac amyloidosis using, for the first time, Implantable Loop Recorders.
- To present the first comprehensive review of Light Chain Deposition Disease highlighting the relationship between haematological response and overall prognosis

Results

In 65% of cases where renal amyloidosis was misdiagnosed as minimal change disease, Congo red staining was not undertaken and in 35% of cases neither Congo red staining, with cross-polarised light visualisation, nor electron microscopy was undertaken.

Proteomics has now been established as a specific and sensitive technique by which to diagnose amyloid and the subtype, demonstrated by distinguishing Fibrinogen Aα-Chain (AFib) renal biopsies from other subtypes.

Urinary RBP/Creatinine (RCR) correlated with the: degree of tubular atrophy, number of light chains, eGFR, presence of glycosuria and degree of tubular phosphate reabsorption. RCR values were especially high in AFib and AA amyloidosis.

Pulseless Electrical Activity was identified as the terminal rhythm in patients with Stage III/IV cardiac amyloidosis and this was preceded by a high degree AV block.

Deep clonal responses to chemotherapy are associated with improved renal and overall outcomes in LCDD and should be pursued even in advanced chronic kidney disease.

Ethical Approval

All individuals whose data has been used in the clinical research studies described in this thesis gave explicit informed consent by signing a consent form whilst visiting the centre. The consent form was approved by the Royal Free Hospital Ethics Committee (REC Ref 06/Q0501/42). The dosage and administration of radioactive isotopes were approved by the Administration of Radioactive Substances Advisory Committee of the Department of Health. Those patients participating in the 'Implantable Loop Recorder Study' signed an additional consent form for device implantation and monitoring.

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Abbreviations

AA	Systemic Amyloid A Amyloidosis
AApoA1	Hereditary Apolipoprotein AI Amyloidosis
AApoAII	Hereditary Apolipoprotein AII Amyloidosis
Aβ2M	β2-Microglobulin Amyloid
ACE	Angiotensin Converting Enzyme
AF	Atrial Fibrillation
AFib	Hereditary Fibrinogen A α-chain Amyloidosis
AGel	Gelsolin Amyloidosis
AL	Light Chain Amyloidosis
ALys	Hereditary Lysozyme Amyloidosis
ALP	Alkaline Phosphatase
ASCT	Autologous Stem Cell Transplantation
ATTRwt	Wild Type Transthyretin Amyloidosis
AV	Atrio-Ventricular
BJP	Bence Jones Proteins
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
BP	Blood Pressure
CVA	Cerebrovascular Accident
CI	Confidence Interval
CKD	Chronic Kidney Disease
CMR	Cardiac Magnetic Resonance imaging
CR	Complete (light chain) Response
CRP	C-Reactive Protein

CPHPC	R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2-carboxylic acid
dFLC	Free Light Chain difference
DNA	Deoxyribonucleic acid
DPD	99mTc-3, 3-diphosphono-1, 2-propanodicarboxylic acid
ECG	Electrocardiogram
ECOG	Eastern Co-operative Group
EDTA	Ethylenediaminetetraacetic Acid
EF	Ejection Fraction
eGFR	Estimated Glomerular Filtration Rate
ESRF	End Stage Renal Failure
FAP	Familial Amyloid Polyneuropathy
FLC	Free Light Chain
FMF	Familial Mediterranean Fever
GI	Gastro-Intestinal
HR	Heart Rate
H_2O_2	Hydrogen Peroxide
ICD	Implantable Cardioverter-Defibrillator
IHC	Immunohistochemistry
ILR	Implantable Loop Recorder
IL-1	Interleukin-1
IL-6	Interleukin-6
IVSd	Interventricular Septal Thickness in Diastole
LGE	Late Gadolinium Enhancement
LV	Left Ventricular
LCs	Light Chains

LCDD	Light Chain Deposition Disease
MCD	Minimal Change Disease
MGUS	Monoclonal Gammopathy of Undetermined Significance
MI	Myocardial Infarction
NHS	National Health Service
NAC	UK National Amyloidosis Centre
NR	No (light chain) Response
NT-proBNP	N- Terminal pro- Brain Natriuretic Peptide
NYHA	New York Heart Association classification
PCD	Plasma Cell Dyscrasia
PCR	Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
PR	Partial (light chain) response
PTC	Proximal Tubular Cells
RA	Rheumatoid Arthritis
RBP	Retinol Binding Protein
SAA	Serum Amyloid A protein
SAP	Serum Amyloid P component
SCD	Sudden Cardiac Death
SD	Standard Deviation
TNF	Tumour Necrosis Factor
TSH	Thyroid Stimulating Hormone
TTR	Transthyretin
TNF	Tumour Necrosis Factor
UCL	University College London
VT	Ventricular Tachycardia

Chapter One

Introduction

This chapter is, in part, based on the publication below:

Emerging treatments for amyloidosis. Sayed RH, Hawkins PN, Lachmann HJ.

Kidney Int. 2015 Mar; 87(3):516-26. Review

The term amyloidosis encompasses a group of diseases caused by the pathogenic misfolding and aggregation of specific proteins, resulting in the formation of insoluble fibrils and their accumulation in the extracellular space, ultimately causing organ dysfunction and death. The term *amyloid* or starch-like was first coined by Virchow in 1858 following the reaction of tissue deposits to iodine and sulfuric acid, this mixture conventionally being used to identify starch in plants [1]. Since then, more than 30 different amyloid fibril precursor proteins have been identified, but all types of amyloid fibril share an essentially similar ultrastructure [2]. This acquired highly characteristic β pleated conformation of amyloid fibrils is associated with specific biophysical properties, including the ability to bind Congo red dye in a spatially ordered fashion that produces diagnostic green birefringence when viewed under cross-polarised light [3, 4]. Under electron microscopy, amyloid deposits appear as randomly arranged, rigid nonbranching fibrils of ~10 nm in diameter and of indeterminate length [5]. All subtypes also contain Serum amyloid P component (SAP) which is a normal serum, non-fibrillar plasma glycoprotein, specifically concentrated in amyloid deposits as it binds avidly and reversibly to all types of amyloid fibrils [6]. This component plays a crucial role in an exciting new treatment strategy for amyloidosis and is also used

for binding purposes in SAP scintigraphy and in confirming amyloid deposition in proteomic analysis [7].

Recent diagnostic developments have improved disease recognition and the incidence of systemic amyloidosis is estimated to be at least *c*.8 per million per year although likely to be higher [8]. Untreated, the prognosis for the commonest amyloid subtype is only 12 months and the prognosis in treated individuals, after 20 years of drug development, now exceeds 3 years [9]. It is estimated to be the cause of death in 0.58/1000 cases and is responsible for 0.8% of cases of end-stage renal disease [8, 10]. It remains critically important that we strive to further lessen the morbidity and mortality burden that accompanies the diagnosis of such a potentially debilitating disease.

Pathogenesis

Essentially there are understood to be 4 main settings in which amyloid formation can occur:

Persistently high levels of a normally present protein due to either increased production, as in AA amyloidosis where serum amyloid A (SAA) levels are increased or high levels secondary to decreased elimination e.g. β2-microglobulin in Aβ2m in end-stage renal failure (ESRF).

Short term exposure to a highly amyloidogenic precursor protein such as light chains (LCs) in light chain (AL) amyloidosis or variant proteins in hereditary amyloidosis.

When there is normal abundance of a ‘normal’, but to some extent inherently amyloidogenic protein over a very prolonged period which undergoes remodelling,

such as transthyretin in wild type TTR amyloidosis (ATTRwt), formerly known as senile systemic amyloidosis.

When the mechanism is as yet unknown e.g. in Leukocyte chemotactic factor 2 amyloidosis (ALECT2), where raised levels, mutations or proteolytic remodelling have not yet been identified as a possible cause [11].

However, clearly only a small percentage of patients in such circumstances go on to develop amyloidosis i.e. not everyone with a long term inflammatory process or plasma cell dyscrasia (PCD) or advancing age will develop AA, AL or ATTRwt (respectively) amyloidosis. Additionally, in the hereditary amyloidosis, even in kindred with the same genetic mutation there can be great variability in penetrance and severity, perhaps related to the size of the precursor protein fragment [12]. Hence there are genetic and environmental factors at play that we do not fully comprehend.

The exact mechanisms by which amyloid deposits, once formed, cause tissue damage have not yet been fully elucidated. It is generally believed that large amounts of insoluble amyloid fibril aggregates deposit extracellularly, disrupt tissue architecture and mechanically interfere with the physiologic function of affected organs [13]. These pathological fibrils arise due to 'misfolding' of the precursor proteins into a β- sheet structure as opposed to the usual α helical structure formed by most proteins [14]. However, the reason for this propensity towards abnormal fibril formation is poorly understood. Additionally, once formed, amyloid fibrils act as a nidus encouraging the exponential accumulation of further amyloid- as demonstrated by experimental mice models in whom amyloid tissue was injected. They developed AA amyloidosis within days of an acute inflammatory stimulus unlike 'unprimed' mice who developed amyloid weeks after an ongoing inflammatory stimulus. Amyloid thus

rapidly accumulates once this amyloid enhancing factor / template is established. Furthermore, prefibrillar oligomeric species may also be toxic and contribute to organ dysfunction; cytoxicity, in these cases, seems to be related to structural flexibility and exposure of hydrophobic residues [15]. Once formed, interactions with other moieties renders the amyloid fibril resistant to degradation, notably glycosaminoglycans, such as heparan and dermatan sulphate, as well as SAP [16]. These promote misfolding and SAA aggregation both *in vitro* and *in vivo* and accelerate the process through acting as a scaffold for polymerisation. Heparan sulfate is thought responsible for the stability of SAA_2, the immediate precursor to AA amyloid, in adopting the β-sheet structure [17]. In the case of transthyretin amyloidosis, the conversion of circulating TTR protein into amyloid requires dissociation of the normal tetrameric protein into monomers, and a massive change in conformation and assembly to form the fibrils [18]. The stability of the tetramer and transition to amyloid are influenced by disease-associated mutations and environmental factors such as pH and oxidative stress [19, 20].

Overall, fibrillation is an unfavourable process in which proteins overcome a thermodynamic and kinetic barrier and, therefore, it is not unexpected that it occurs over a long time [21]. Increasing evidence supports the possibility that multiple, morphologically distinct forms of amyloid fibrils may be generated from a single amyloidogenic protein through different aggregation pathways. Some of the risk factors leading to the formation of pathological, misfolded and insoluble fibres are known to us: time and a sustained, critical concentration of the precursor protein, changes in amino acids leading to variant protein production as seen in the hereditary amyloidosis and proteolytic remodelling of the protein precursor, as in the case of β-amyloid precursor protein in Alzheimer's disease [22]. These mechanisms can occur independently or in association with one another.

Amyloid regression has occasionally been seen post treatment, as evidenced by functional testing, imaging and blood tests. The exact mechanism is unknown but macrophages and the complement pathway are both believed to play a role, with the former identified around amyloid deposits [23]. Studies suggest that macrophages have both a role in the formation and the degradation of amyloid deposits. In amyloid AA and AL amyloid, the precursor protein is proteolysed by macrophages prior to its deposition as an amyloid fibril and hence macrophages are involved in amyloid formation. With regards to degradation, macrophages form multinucleate giant cells whose increased surface area, arising from membrane ruffles, aids in the phagocytosis of large C3 opsonised objects [23]. Macrophage depletion with liposomal clodronate, has been shown to retard amyloid regression *in vivo* [24]. It may be that differences in rates of amyloid regression are secondary to differences in the phenotype and function of macrophages or monocytes, as well as the success of treatment in suppressing amyloidogenic precursor protein production. The net effect of amyloid regression is stabilisation or improvement of organ function coupled with increased patient survival.

Amyloid Subtypes

As mentioned above there are >30 different types of fibril precursor protein giving rise to varying amyloid subtypes, although not all cause overt disease. The current nomenclature consists of the first letter, A (for amyloid), followed by a description of the precursor protein e.g. primary or AL associated amyloidosis which arises from a PCD. The subsequent disorders can be divided according to distribution type (systemic/ localised) or depending upon whether they are acquired or hereditary. The more common of these are listed in Table 1.1, followed by a brief description of some of the subtypes.

Table 1.1. The major amyloid subtypes

Amyloid Subtype	Fibril Precursor	Treatment	Organs Involved
AL	Monoclonal free immunoglobulin light chains	Chemotherapy directed at the underlying plasma cell dyscrasia Potentially novel agents	Renal (50-80%), cardiac, liver, spleen, bones, GI, autonomic and peripheral neuropathy, soft tissue
AA	Serum amyloid A protein	Treatment aimed at specific underlying inflammatory condition Potentially eprodisate	Renal (>95%), liver, spleen, adrenals, autonomic neuropathy
TTR: **Senile Systemic** TTR: **Hereditary**	Wild type transthyretin Variant transthyretin (more than 100 different variants)	Cardiac transplantation Potentially novel agents Liver +/- cardiac +/-renal transplantation Tafamadis Potentially novel agents	Cardiac, soft tissue Dominant neurological+/- cardiac involvement (dependent upon specific TTR variant)
A Fib (hereditary)	Variant Fibrinogen Aα	Renal ± liver transplantation Potentially novel agents	Renal

Apolipoprotein AI (hereditary)	Variant Apolipoprotein AI	Renal +/- Liver transplantation Potentially novel agents	Renal (mainly medullary), liver, heart, skin, larynx
Apolipoprotein AII (hereditary)	Variant Apolipoprotein AII	Renal transplantation Potentially novel agents	Renal
AGel	Variant gelsolin	Renal transplantation	Cranial nerve. involvement Lattice corneal dystrophy
Lysozyme (hereditary)	Variant lysozyme	Renal transplantation Potentially novel agents	Renal, liver, GI, spleen, lymph nodes, lung, thyroid, salivary glands

Abbreviations: AA, amyloid A; AFib, fibrinogen Aα-chain; AL, immunoglobulin light chain amyloidosis; ATTR, amyloidogenic transthyretin; β2M, β2-microglobulin; GI, gastrointestinal; TTR, transthyretin

Systemic Amyloidosis

AL amyloidosis

AL amyloidosis, otherwise known as immunoglobulin LC amyloidosis, taking its name from the amyloidogenic LCs, more commonly λ than κ, which are almost invariably produced from an underlying B-cell dyscrasia [25]. Rarely, a combination of heavy and LCs cause the disease, or just heavy chains alone can cause systemic amyloidosis, termed AHL and AH amyloidosis respectively [26]. The LCs themselves, which are most commonly part of the variable domain, are inherently pathogenic and propagate fibril formation leading to systemic amyloidosis [27]. There is evidence of differences in the amino acid substitution in this variable region between those patients with a PCD who go on to develop amyloidosis and those patients who do not [28] . The most severe manifestation is cardiac amyloidosis, occurring to some degree in >70% of patients and largely determining both treatment tolerance and outcome [29]. This most commonly manifests as a restrictive cardiomyopathy ultimately resulting in congestive cardiac failure, at which point median survival is only 4-6 months [30]. A cohort of patients thus affected are investigated further in the Implantable Loop Recorder Study, as described in Chapter 6. Equally frequently the kidneys have also been found to be affected which gives rise to proteinuria, progressing in some patients to nephrotic syndrome [31]. The pathognomonic periorbital purpura and macroglossia actually occurs in only <15% of patients with systems, such as the hepatic (22%), autonomic and peripheral nervous system (15%) and soft tissues (15%) being involved more commonly [32]. The brain is not affected.

AA Amyloidosis

In AA amyloidosis, the amyloid deposits are derived from the circulating acute phase protein, serum amyloid A (SAA), an apolipoprotein of HDL type, whose N-terminal fragments spanning the first 66-76 amino acids form the actual protein precursors [33]. SAA levels range between 1 to >3,000 mg/L, with higher levels occurring when production is upregulated to potentially a 1000-fold during acute inflammation [34, 35]. SAA is synthesised by hepatocytes under regulatory control of proinflammatory cytokines such as tumour necrosis factor (TNF), interleukin 1 (IL-1), interleukin 6 (IL-6) and transcription factors such as SAA activating factor and lipopolysaccharide [36-38]. Additionally, the hepatic clearance of SAA during both acute and chronic inflammation is reduced, adding to the elevated levels seen [39]. Although SAA's exact function as an acute phase reactant remains unclear, persistently elevated SAA levels are a ubiquitous feature noted in all established human cases of SAA amyloidosis.

The onset of organ dysfunction is usually acute, suggesting the presence of a triggering event in patients who do develop amyloidosis; the major target organs involved are the spleen and kidneys with progressive proteinuric renal dysfunction being the most clinically relevant common presenting feature with approximately 25% of cases progressing to ESRF. Cardiac involvement and neuropathy are only ever very rarely reported and hepatic involvement, a feature of advanced disease, confers a poor prognosis [40].

In the developed world, the most frequent predisposing conditions are the inflammatory arthritides, followed by chronic sepsis, inflammatory bowel disease and the hereditary periodic fever syndromes (Table 1.2): Familial Mediterranean Fever (FMF), hyper-immunoglobulin D syndrome (HIDS), TNF receptor associated periodic fever syndrome (TRAPS), familial cold urticaria (FCU) and Muckle-Wells syndrome (MWS) [40].

Tuberculosis still remains the commonest cause overall although AA amyloidosis may occur in association with any kind of chronic inflammatory disorder and the lifetime incidence of AA amyloidosis in those with chronic inflammatory conditions is 1-5%. In 6% of patients there is no clinically evident underlying inflammatory disorder [40, 41].

Table 1.2. Inflammatory disorders potentially resulting in SAA protein amyloidosis

Disorder
Chronic Inflammatory Disorder:
Rheumatoid arthritis
Juvenile Inflammatory Arthritis
Ankylosing Spondylitis
Psoriasis and Psoriatic Arthropathy
Reiter's Syndrome
Adult Still's Disease
Behcet's Syndrome
Crohn's Disease
Ulcerative Colitis
Gout
Chronic Microbial Infections
Leprosy
Tuberculosis
Bronchiectasis
Cystic Fibrosis
Decubitus ulcers

Complications of paraplegia

Osteomyelitis

Whipple's Disease

Infections in intravenous drug abusers

Ciliopathies

Neoplastic Disease

Hodgkin's lymphoma

Non-Hodgkin's Lymphoma

Renal carcinoma

Carcinomas of gut, lung, urogenital tract

Basal Cell Carcinoma

Hairy Cell Leukaemia

Castleman's Syndrome

Autoinflammatory Diseases

Familial Mediterranean Fever

TNF receptor-associated periodic fever syndrome

Hyper-IgD syndrome

Cryopyrin-associated periodic syndrome

Familial Cold Autoinflammatory Syndrome

Muckle-Wells Syndrome

Neonatal-onset multisystem inflammatory disease

Mevalonate kinase deficiency

Schnitzler Syndrome

Systemic Vasculitis

Behcet's Disease

Polyarteritis Nodosa

Giant cell arteritis

Takayasu's arteritis

Polymyalgia Rheumatica

Others

? Obesity

Sarcoidosis

SAPHO syndrome *(synovitis, acne, pustulosis, hyperostosis, osteitis)*

Although the median latency between onset of inflammation and diagnosis is approximately 17 years, some individuals develop amyloid within months [40]. Outcome in AA amyloidosis is favourable when SAA concentrations remain below 10mg/l and correspondingly, it is the control of inflammation and hence of SAA production in patients with AA amyloidosis which arrests amyloid accumulation [42]. Although outcomes have improved markedly in recent years through better control of inflammation, AA amyloidosis is still a potentially fatal complication of chronic inflammatory states and there remain important lacunae in our understanding of its pathogenesis, including: the factors which predispose only certain individuals to developing amyloidosis, the exact mechanism by which inflammation leads to amyloid deposition and why the distribution, severity and progression of amyloid deposition differs in those affected.

Transthyretin (ATTR) Amyloidosis

Transthyretin (TTR) is a 55kDa homotetrameric plasma protein almost exclusively produced from the liver, save for 5% from the choroid plexus and retina. It *trans*ports *thy*roxine and *retin*ol (hence *transthyretin)* and is associated, in its wild-type (wt) form, with acquired amyloidosis of the senile systemic type, now termed wild-type transthyretin amyloidosis or ATTRwt [43]. More than 100 genetic variants of TTR are associated with autosomal dominant hereditary amyloidosis, the most common inherited amyloidosis worldwide, and these mutations may increase TTR's predisposition to misfold into an insoluble β-pleated sheet.

Wild-type transthyretin (ATTRwt) amyloid

ATTRwt amyloidosis is being ever more increasingly diagnosed and is an important differential in elderly patients with putative hypertensive or hypertrophic cardiomyopathy, especially in cases where there is heart failure with a preserved ejection fraction. ATTRwt can be associated with hypotension, aortic stenosis with a paradoxical slow flow and atrial fibrillation [44-46]. It is much less rapid in progression than AL or AA amyloidosis, but is more common, especially in elderly white men with a 25 to 50:1 male: female preponderance [47]. Approximately 25% of patients >80 years had amyloid detected in a cardiac autopsy study, although only two-thirds of these patients actually had left ventricular involvement and 50% of these had any significant level of involvement- placing the prevalence at 8-16% [47-49] . ATTRwt presents as restrictive cardiomyopathy, congestive cardiac failure, arrhythmias and/or conduction defects with patients having a better median survival than that in cardiac AL amyloidosis [50]. Cardiac symptoms are preceded by a history of carpal tunnel syndrome, with the incidence being 48% in one study and onset a median of 8 years prior

to the diagnosis of heart failure; amyloid deposits have been found on palmer fascia biopsies post carpal tunnel release surgery [51]. 4% of patients presented with haematuria and evidence of bladder amyloid, with other non-clinically significant deposits in ATTRwt frequently being present in the lungs, gut and small arteries [52].

A left bundle branch pattern on ECG is seen more commonly in ATTRwt and small QRS complexes are seen in other subtypes of cardiac amyloid too [53]. Echocardiography demonstrates a markedly thickened myocardium, more so than that typically found in cardiac AL amyloidosis [54]. In a study of 18 patients with ATTRwt the mean interventricular septal thickness was 17.8mm in the ATTRwt group as compared to 14.3mm in the AL group (P=0.002) [47]. The identification of ATTRwt has lately increased due to the advent of CMR imaging but diagnosing the exact amyloid subtype on CMR alone is difficult. Although 99mTc-DPD scintigraphy is a sensitive imaging technique in diagnosing cardiac transthyretin amyloidosis especially where Perugini high grade imaging is present, there are cases of DPD positive scans in AL amyloidosis [55]. Such cases cannot be differentiated through biomarkers alone i.e. 7.5% of patients >85 years old have a monoclonal gammopathy of uncertain significance (MGUS) so serum LC aberration cannot be assumed to signify AL amyloidosis [56]. Hence, the definitive diagnosis of transthyretin amyloid continues to rest on the combination of finding transthyretin amyloid on endomyocardial biopsy and wild-type TTR gene sequencing [57, 58]. The correct diagnosis is crucial as management differs markedly from AL cardiac amyloid i.e. the mainstay of management is supportive care and symptomatic management in ATTRwt amyloid as opposed to potentially chemotherapy or stem cell transplantation in AL amyloidosis. In two prospective studies the median survival time from diagnosis was 43 and 46.7 months, with 78% of the deaths in the latter being secondary to cardiac causes [47, 59, 60]. A recent study of 121 patients with

ATTRwt identified uric acid, Brain Natriuretic Peptide (BNP), left ventricular ejection fraction (LVEF), and relative wall thickness as predictors of shorter survival [60].

Hereditary Transthyretin Amyloidosis

Hereditary TTR amyloidosis mostly affects the peripheral and autonomic nervous system and/or the heart, termed Familial Amyloid Polyneuropathy (FAP) and Familial Amyloid Cardiomyopathy (FAC) respectively [61, 62]. Depending upon the mutation, the: penetrance, presence, age of onset, severity and extent of cardiac and neuropathic involvement differs. Additionally, there are varying degrees of amyloid deposition in the viscera, vitreous, gut, and occasionally the central nervous system. Typically, the neuropathy is a length dependent ascending, sensorimotor, axonal polyneuropathy; autonomic involvement is usually present, manifesting as: orthostatic hypotension, dysfunctional sweating, a change in bowel habit, erectile dysfunction, urinary incontinence and orthostatic syncope [63]. Untreated FAP is a progressive disease resulting in death within 7-15 years; although renal amyloid deposits do occur, only 34.6% develop chronic kidney disease (CKD) and 10% progress to ESRF [64, 65]. Notable variants are as follows:

Met30 TTR variant

This is the commonest transthyretin mutation globally, arising from the substitution of methionine for valine at position 30 (ATTRV30M) and is characterised by a progressively debilitating and painful sensorimotor neuropathy and autonomic involvement with cardiac involvement being rare. Onset is usually by 30-40 years in the Portuguese cohort, but about 20 years later in the Swedish one and death occurs a median of 10 years post symptom onset [66-68].

T60A TTR Variant

This is the commonest cause of FAP in the UK and Ireland, usually presenting after the age of 50 with autonomic symptoms, but cardiac amyloid is virtually always present at diagnosis [69]. It is believed to originate from northwest Ireland, specifically Donegal [70].

V122I Variant

3-4% of African-Americans have the V122I transthyretin variant, which is associated with a predominantly cardiac phenotype without a neuropathic component, usually presenting after the age of 60 and clinically indistinguishable from ATTRwt [71, 72] . It does not actually contribute to excess mortality but does contribute to excess morbidity, in that there is a higher incidence (10%) of New York Heart Association Classification III and IV [73]. The mutation is thought to have originated from the Caribbean [58].

Transplantation in Hereditary TTR Amyloidosis

As >90% of circulating TTR protein is liver derived, ‘surgical gene therapy’ with orthotopic liver transplantation has been undertaken in more than 2000 FAP patients since 1990 [74, 75]. Liver transplantation when performed early has been shown to prolong life, particularly in patients with the TTR Met30 variant, which makes up 83% of transplant cases, but long-term outcomes reveal that neuropathy and organ involvement are not typically reversed [76, 77]. BMI, early disease onset (age <50yrs), disease duration pre- transplantation and TTR V30M versus non-V30M TTR mutations are independent and significant factors affecting survival [63]. Post-transplantation, patients with V30M mutations have a 5year survival rate of 100% as opposed to 59% for patients with non-V30M ATTR [75]. This may reflect the occurrence of amyloid cardiomyopathy post-operatively, thought to be due to the deposition of wt transthyretin on a template of pre-existing cardiac amyloid derived from variant proteins [78]. In fact, renal, neuropathic and or GI deposits have also been found to contain both wt

and variant TTR amyloid in mutant TTR carriers, and in patients, post liver transplantation, the ratio of wt to mutant TTR increases implying that 'pathological' wtTTR continues to deposit in affected tissues post- transplant in a time-dependent manner and leads to a deterioration in quality of life ~4 years post liver transplantation [79]. The clear need for other therapies has resulted in the development of novel anti-amyloid therapies, both with a view to stabilising soluble TTR in the blood i.e. tafamadis and diflunisal which have already been proven effective in FAP and are used by several hundred patients, and by inhibiting its production through silencing RNA and anti-sense oligonucleotide approaches [80]. Several strategies have already progressed to clinical trial [81].

Hereditary non- ATTR Systemic Amyloidosis

There are several forms of hereditary systemic amyloidosis, which are associated with genetically variant proteins, mostly comprising single amino acid substitutions. They are inherited in an autosomal dominant manner with variable penetrance accounting for a frequent absence of a family history, suggesting a contributory role of other factors [82].

Hereditary Gelsolin Amyloidosis (AGel)

Hereditary gelsolin amyloidosis otherwise known as familial amyloidosis of the Finnish, is an autosomal dominant disorder secondary to at least 2 known mutations: guanine's replacement by adenosine on chromosome 9 at q33.2, GSN c.640G > A, and GSN C.640G>T [83]. The former was first described and believed to be most common in the Finnish population where it is one of the most common hereditary disorders. It has now been identified in North America, Asia and Mexico, whilst GSN C.640G>T has been found in Denmark, former Czechoslovakia, France and Brazil [84].

Gelsolin itself is a calcium-activated, actin-modulating protein [85]. AGel amyloid fibres are believed to accumulate in various tissues with a triad of clinical sequelae: progressive corneal lattice dystrophy, a cranial bifacial neuropathy (amongst other neurology) and signs of cutix laxa [86]. Other less common features include potentially fatal generalised amyloid angiopathy, renal amyloidosis (nephrotic syndrome), conduction defects, valvulopathies, depression and progressive cranial neuropathy leading to bulbar palsy and oropharyngeal tissue laxity [87-91]. The age of onset can be as early as 13 years in homozygous disorders and around the age of 30 in heterozygous disorders. Although penetrance is 100%, disease manifestations and severity vary between patients, with the diagnosis being verified using molecular genetic analysis [84]. In a recent study, 20% of 272 deaths were attributed to AGel, although the exact mechanism was not found and interestingly cancer as a cause of death was less frequent than in the general population and renal complications were more frequently fatal, especially in females [92]. There is no specific curative treatment at present but symptomatic treatments to improve quality of life include: good ophthalmological care and lubrication, corneal transplantation, surgical intervention to correct facial laxity, and renal transplantation [86, 93].

Fibrinogen A α-chain (FibA) Amyloidosis

Renal amyloid composed of fibrinogen A α-chain is the most common form of hereditary renal amyloidosis worldwide and was first identified in 1993 in a Peruvian family [94]. Fibrinogen is a liver-derived plasma glycoprotein that plays an essential role in the coagulation pathway. It consists of 2 identical sets of 3 polypeptide chains termed α, β, and γ, joined by disulfide bridging. Each polypeptide is encoded by a distinct gene and known as Fibrinogen alpha (FGA), beta (FGB) and gamma (FGG). The gene for the fibrinogen A α-chain which has 610 amino acid residues is localised on chromosome 4 and has 6 exons. Mutations in any of the 3 genes encoding for fibrinogen polypeptides can cause

dysfibrinogenemias, and mutations in the A α-chain gene can lead to hereditary systemic amyloidosis [95]. The diagnoses FibA amyloidosis, including the different variants, using proteomics is further discussed in Chapter 4 of this thesis.

Patients with AFib amyloidosis typically present with nephrotic syndrome and hypertension, usually in the 5th decade of life [96]. CKD usually develops within 1-5 (median 4.6) years from the time of diagnosis. Patients with ESRD have an estimated survival time of approximately 9 years and survival from the time of presentation is typically 15 years. Renal transplantation has been undertaken but graft survival, on average, is 6.7 years and failure occurs due to recurrent disease [94]. In comparison, combined liver and kidney transplantation, in which the circulating amyloidogenic variant has been removed, is a curative operation and removes the risk of allograft failure due to recurrence of amyloid. However, despite careful patient selection there was not an inconsiderable amount of morbidity and mortality associated with a combined liver-kidney transplant with a procedure related mortality of 33% [94].

Leukocyte Chemotactic Factor 2 Amyloidosis (ALECT2)

ALECT2 is believed to be hereditary although the exact mutation has not yet been identified [11]. It is a familial disorder and one which has been found to occur most frequently in certain population groups, including Hispanic, Punjabi, Arab, Israelis, Native Americans and Sudanese [97, 98]. It is the third most common cause of hereditary renal amyloidosis and second commonest cause of hepatic amyloidosis. The median age of diagnosis in the largest case series was 70.4 years and a progressive decline in renal function was noted with an eGFR deterioration of 0.5 ml/min/1.73 m^2 per month [99]. Heavy proteinuria was not a feature unless there was a co-existing glomerulopathy and most patients had proteinuria <200mg/day [99]. From the 3 largest studies, proteinuria was absent in up to 67% of cases

[98-100]. ALECT2 deposits are very Congophilic and widespread, save for the medulla, with deposits being noted in the renal cortex, glomeruli and arteries.- unlike APOA IV which has a medullary preponderance [99]. 26 of the 40 patients in this case series underwent gene sequencing and all 26% were homozygous for the G nucleotide in a non-synonymous SNP at position 172 (SNP rs31517). However, this polymorphism is common in those with a Mexican heritage and so does not necessarily imply causality, however the authors concluded that there was another, as yet unknown, mutation which, combined or having aggregated with the aforementioned polymorphism, led to the ALECT2 phenotype [101]. Serum LECT2 concentrations were not shown to be elevated, suggesting that systemic overexpression of the LECT2 protein is not the aetiology. There is no specific treatment for ALECT2, although it is important to accurately diagnose this type of amyloid to avoid mismanagement of ALECT2 as though it were AL amyloidosis [101]. Patients have undergone renal transplants with a good short-term outcome thus far [102].

Localised Amyloidosis

Localised amyloidosis refers to amyloid deposition confined to a single organ or tissue which will not evolve into systemic amyloidosis and so it is particularly important to ensure that it is not an early manifestation of the relatively more common systemic AL. These local deposits are believed to be associated with LCs, as identified by immunohistochemistry, arising from a focal B cell dyscrasia [103]. Case reports and the largest retrospective cohort study to date (606 patients) has found that most of the sites affected are mucosal in nature and include (in order of frequency of occurrence): bladder, laryngeal or tonsillar, cutaneous, pulmonary nodular, gastrointestinal, oral, tracheobronchial, lymph node, conjunctival, bone, eyelid, orbital, breast, ureteric, urethral, soft tissue, prostate, cerebral and aortic valve in one (<1%). Assessment also included gene sequencing if, for example laryngeal amyloid was suspected as this is associated with APOA 1 mutations and TTR gene sequencing where

samples stained for transthyretin, to exclude ATTR amyloidoisis (1% of bladder biopsies) [104]. Although follow up studies have not revealed evolution into systemic amyloidosis over 20 years, it is important to follow up such patients to ensure that the initial diagnosis is correct as 1% of patients have actually been found to have systemic disease and required chemotherapy [104]. Additionally, follow up is important as patients require assessment of local symptoms and sometimes treatment i.e. 51% of patients needed an intervention and 21% of them needed more than one. Treatment in these cases was for pressure effect and pain and comprised of surgical resection and/or radiotherapy. Diagnosis usually takes 7 months from the time of medical presentation with a median age of presentation of 59.5 years and no gender bias; life expectancy is not affected by this diagnosis [104].

Symptoms, Signs and Assessment

The insidious and diverse nature of symptoms arising from amyloidosis which may well overlap with any number of disease processes, can make diagnosing amyloidosis difficult at times- and yet there are some patients who present without any of the overt signs of amyloidosis. The sequelae of amyloidosis, regardless of type, arise from the infiltration of that organ by amyloid fibrils. The textbook patient would be one who presents with bilateral peri-orbital bruising, macroglossia, carpal tunnel syndrome or another neuropathy, dyspnoea and swollen lower limbs- however this constellation of symptoms is not usually seen. Additionally, phenotype can vary widely not only between the differing forms of hereditary amyloid but also within the same genetic disorders i.e. age of onset and severity can vary between family members, with females being affected later and less severely. The number and combination of different tissues affected differs between different subtypes and also amyloid can occur as a localised disease, restricted to one particular tissue [67]. There

is no evidence that amyloid deposition occurs in the brain [105]. The table below summarises the different symptoms and signs arising from involvement of various tissues and the usual mode of assessment and diagnosis. The statements in bold refer to diagnostic features in systemic AL as proposed by the 10th International Symposium in Amyloid and Amyloidosis, importantly a histological diagnosis is essential in every case [106].

Table 1.3 Organ specific symptoms and signs associated with amyloid deposition

Tissue Involved	**Symptoms & Signs***	**Diagnosis & Assessment**
Cardiac	Dyspnoea Peripheral and pulmonary oedema Pleural and pericardial effusions Atrial and/or ventricular arrhythmias Hypotension Syncope Sudden death	6-minute walk test Biomarkers: Nt-proBNP cTnT ECG +/- Holter monitoring DPD scan **Echo: mean wall thickness > 12 mm, no other cardiac cause** CMR **Endomyocardial biopsy**
Coagulation cascade	Periorbital purpurae Bruising	Testing for Factor X and IX deficiencies [107]
Eyes	Vitreous amyloid deposits (localised and in some variant TTRs) Extraocular muscle involvement (ptosis, ocular motility disturbances) Lacrimal gland (keratoconjunctivitis sicca) Orbital fat- palpable mass lesions	Biopsy

	Corneal lesions/ Lattice corneal dystrophy- visual disturbances, painful erosions Conjunctiva- painless yellow, pink rubbery lesion, haemorrhage	
Lung	Dyspnoea Haemoptysis	**Direct biopsy verification with symptoms** **Interstitial radiographic pattern in systemic AL**
Hepatic	Right upper quadrant fullness Bruising Peripheral oedema (low albumin) Jaundice Can progress to liver failure	Elasticity Clotting, transaminases **Radionuclide imaging/CT:** **Total liver span > 15 cm in the absence of heart failure or alkaline phosphatase > 1.5 times institutional upper limit of normal** **Biopsy**
GI tract	Motility disturbances secondary to autonomic nervous system involvement Malabsorption Altered bowel habit GI haemorrhage	**Biopsy of affected site**
Palmer fascia	Carpal tunnel syndrome	EMG
Skin	Bruising Lichen amyloid- pruritis Macular amyloid Nodular localised cutaneous amyloidosis	Biopsy

Connective tissue	**Pseudohypertrophy** of skeletal muscle, **arthropathy**, **carpal tunnel syndrome, lymph node (s)**	Biopsy: **myopathy**
Tongue	**Macroglossia**: dysphagia, dysphonia obstructive Sleep Apnoea	
Nervous System	Peripheral neuropathy (17-35% of AL amyloidosis patients and majority of TTR cases)- length dependent thermanesthesia, autonomic symptoms e.g. orthostatic hypotension, impotence, urinary retention and GI dysfunction and burning neuropathic pain. In advanced cases this can progress to a motor neuropathy [108-110] Gelsolin [111] Apolipoprotein A1 [112]	**Clinical examination findings:** **Peripheral: clinical; symmetric lower extremity sensorimotor peripheral neuropathy** **Autonomic: gastric-emptying disorder, pseudo-obstruction, voiding dysfunction not related to direct organ infiltration**
Renal	Uraemic symptoms Froth in urine	Serum creatinine/eGFR **24-hr urine protein > 0.5 g/day, predominantly albumin**

*not all or none may be present depending on severity and site of lesion

Statements in bold refer to diagnostic criteria as per Gertz et al. for AL amyloidosis [106]

Investigations

Investigations are more extensively described in the materials and methods' section. This is an explanation for the rationale behind the investigations performed.

Investigation for an Underlying Plasma Cell Disorder

The diagnosis of AL amyloid requires both evidence of amyloid deposition on biopsy and also the demonstration of an underlying PCD. An increase in serum FLC levels precedes the development of AL amyloidosis by many years [113]. An evaluation for suspected amyloidosis should therefore include serum and urine immunofixation electrophoresis to detect monoclonal proteins and a FLC assay to determine the ratio of λ:κ light chains. Circulating FLCs are now quantified by a nephelometric assay (Freelite, The Binding Site, Birmingham, UK) which is reported to be >10 times more sensitive than immunofixation electrophoresis; this quantification is part of the revised Mayo Clinic staging system, which is widely used for risk stratifying patients to certain/any therapy, in clinical trials and also for monitoring response to treatment [114-117]. Evidence for an underlying PCD was detected in 99.8% of patients seen at the NAC [118]. A bone marrow biopsy is still required in AL amyloidosis, to assess the plasma cell burden and to exclude multiple myeloma, in conjunction with a skeletal survey. The presence of a PCD without biopsy, would not serve as definitive evidence of amyloidosis alone, as previously mentioned.

Cardiac Investigations

Biomarkers

In AL amyloidosis, most causes of death are cardiac related and cardiac involvement determines prognosis and management to a large extent i.e. a patient with significant cardiac amyloid would not be suitable for autologous stem cell transplantation (ASCT) and the prognosis in amyloid subtypes that do not affect the heart is much better as compared

to those patients with cardiac amyloid. Hence, a risk stratification system known as the Mayo Clinic (after where it was first devised) criteria using cardiac biomarkers and the difference between the involved and the uninvolved FLC (dFLC) is used for management, prognostic and research standardisation purposes [119]. The two cardiac biomarkers in use are serum cardiac troponin T (TnT) and Brain Natriuretic Peptide (BNP). TnT is a sensitive and specific marker for myocyte injury and is found to be abnormal in >90% of cardiac AL patients [120]. ProBNP is made by myocytes in response to increased wall stress. This 108-amino acid propeptide is produced predominantly in the left ventricle, and when released, is cleaved into two fragments: the active BNP (amino acids 77 to 108) and a leader sequence known as NT-proBNP (amino acids 1 to 76) [121]. The latter has been shown to be a sensitive indicator of cardiac abnormalities. The diagnostic and prognostic significance of both TnT and NT-proBNP has been more widely investigated in AL amyloidosis as opposed to other subtypes, such as wtTTR, and elevated levels are associated with increased mortality i.e. an NT-proBNP level >152 pmol/L as opposed to <152pmol/L is associated with a mortality rate of 72% vs. 7.6% per 100-person years (Table 1.4) [122].

Table 1.4 Risk stratification in AL amyloidosis

Mayo Stage	**cTnT (mcg/L)**	**NT-proBNP (ng/L)**	**dFLC mg/dL**	**5-year Survival (%) [119]**
I	<0.035	<332	<18	59
II	<0.035 Or ≥0.035	≥332 <332	<18	42
III	≥0.035	≥332	<18	20
IV	≥0.035	≥332	≥18	14

Abbreviations: cTnT: cardiac Troponin T, dFLC: difference between the involved and uninvolved free light chains, NT-proBNP: N-terminal pro-Brain Natriuretic Peptide

One third of patients with AL amyloidosis present with cardiac failure associated with marked elevation of both NT-proBNP and TnT concentration and these are the patients who were investigated further in Chapter 6 [123]. BNP/NT-proBNP may also have a local effect with BNP granules being found in high concentrations in myocytes adjacent to amyloid deposits. NT-proBNP's concentration in AL amyloidosis may fall dramatically within weeks following successful chemotherapy, as reflected by a fall in LCs and this is associated with improved outcomes [124]. An early transient increase in BNP/NT-proBNP may occur after treatment frequently used in the management of AL amyloidosis, thalidomide and lenalidomide, but the significance and cause of this are unclear [125].

Electrocardiogram (ECG)

ECG patterns can aid differentiation between AL and TTR amyloidosis and also between cardiac amyloid and hypertrophic cardiomyopathy. The most common finding is low voltage QRS complexes, defined by all limb leads being <5mm in height and this is usually associated with extreme right- or left axis deviation and more commonly seen in AL (46-60%) rather than wtTTR (25-40%) amyloidosis [53, 126]. Poor R wave progression in the chest leads, is present in up to 50% of patients with cardiac AL amyloidosis and a pseudoinfarct pattern is seen equally frequently in AL and TTR (47-69%) amyloid [53, 126]. Left (more common in wtTTR, rare in AL) and right bundle branch block can also occur. The most commonly seen conduction defects are: first-degree atrioventricular block (21%), nonspecific intraventricular conduction delay (16%), second- or third-degree atrioventricular block (3%), atrial fibrillation/flutter (20%), and ventricular tachycardia (5%) [127]. These findings are reflected in the cohort of patients studied in Chapter 6. ECG findings, specifically the combination of R wave voltage of I, avR and QRS was of diagnostic value in differentiating cardiac amyloid from non-obstructive hypertrophic cardiomyopathy [128].

Holter ECG monitoring identifies asymptomatic arrhythmias in >75% of cardiac AL patients (mainly supraventricular tachyarrhythmias and some nonsustained ventricular tachycardia). Congestive cardiac failure, echocardiographic and holter abnormalities have been found to adversely affect survival [129].

Echocardiography & Cardiac MRI

Echocardiography has traditionally been used in the assessment of cardiac amyloidosis and is more recently being used in conjunction with cardiovascular magnetic resonance (CMR), although the latter is restricted to specialist centres with specific operators and is contraindicated in certain subsets of patients e.g. those with severe renal failure and most patients with implanted pacemakers and ICDs [130]. Echocardiography remains better at identifying diastolic dysfunction, which is one of the earliest signs of cardiac amyloid and often precedes symptoms although findings are operator dependent and less precise than in CMR imaging [58]. The granular/ speckled appearance characteristically described is indicative of increased myocardial and valvular echogenicity secondary to amyloid infiltration, although sensitivity for cardiac amyloidosis depends upon machine settings [58].

Cardiac MRI is more useful than conventional echocardiography in assessing systolic function, delineating cardiac amyloid from other causes of hypertrophic conditions e.g. hypertensive, hypertrophic and uraemic cardiomyopathy and storage disorders and producing a more precise 3-dimensional image. CMR also provides diagnostic and prognostic information through the kinetics of global, delayed subendocardial gadolinium enhancement and late transmural gadolinium enhancement. The gadolinium is distributed into the extracellular space, which is greatly expanded secondary to amyloid fibril infiltration, in fact more so than any other cardiomyopathy and these findings have been found to correlate with cardiac biopsies [131]. TI, a CMR parameter used to measure the intrinsic

myocardial signal in mapping studies, has been used to quantify this expansion and serial measures are used to track changes. Equilibrium contrast CMR (EQ-CMR) is a relatively new technique where the equilibrium reached between the amounts of gadolinium in the myocardial interstitium and that in the plasma, allows a numerical estimation of the myocardial interstitial volume. EQ-CMR may detect amyloid infiltration earlier than conventional MRI and can potentially provide a direct measure of the amyloid burden with scope for use in early diagnosis and disease monitoring [132]. Recently transmural late gadolinium enhancement (LGE) has been found to be an independent risk factor for death, as imaged using the technique of phase-sensitive inversion recovery. This is a LGE image reconstruction technique which has been shown to be more sensitive and specific in identifying cardiac involvement and potentially improving risk stratification [133]. This technique obviates the need for determining the optimal 'null point' – the point at which a black and unenhanced myocardium (a normal myocardium) is reached, which is vulnerable to operator error i.e. the abnormal rather than the normal myocardium may be nulled resulting in false negative LGE patterns [134]. However, arrhythmias especially atrial fibrillation and ectopic beats degrade CMR image quality and early cardiac amyloid particularly may only show atypical and patchy LGE, making diagnosis difficult [135].

Typical findings in cardiac amyloid, in both echocardiography and CMR include:

- Biatrial dilatation
- Biventricular, valvular, and interatrial and ventricular septal thickening, especially in wtTTR [136].
- Concentric ventricular thickening with right ventricular involvement, poor biventricular long-axis function with normal/near-normal ejection fraction [137]

- Much greater restriction of basal strain compared to apical movement as opposed to hypertrophic cardiomyopathy. The mean LV basal strain is an independent predictor of both cardiac and overall deaths [125]
- An intracardiac thrombus in 33% of cases of AL cardiac amyloid [138, 139]

Renal Biomarkers

As with other organ biomarkers, these have mainly been studied in the commonest amyloidosis, AL amyloidosis, where renal involvement occurs in 70% of patients [31]. Accurate risk stratification using biomarkers is of great importance in ensuring that the risks of therapy do not outweigh the benefits and precipitate/ hasten renal failure.

Currently used biomarkers are creatinine, modified eGFR and proteinuria. Palladini et al. have recently devised the only widely known renal risk stratification system which found that proteinuria and eGFR independently predict renal survival. Patients with either (Stage II) or both (Stage III) proteinuria >5g/24hrs and eGFR ≥50 mL/min per 1.73 m^2 were found to have a 3-year dialysis requirement of 7-30% and 60-85% respectively. If neither of these risk factors was present, the 3-year progression risk to dialysis was 0-4% [116]. The Mayo group also found that a >75% reduction in proteinuria at 1 year was associated with longer patient survival and an even greater advantage found once proteinuria had reduced by >95% [140].

There are a number of new biomarkers being identified and investigated, in the hope that a more sensitive and specific marker of renal disease, as compared to creatinine, may be found. One such potential biomarker, Retinol Binding Protein, is investigated further in Chapter 5.

Imaging

Serum Amyloid P Component Scintigraphy

This was first developed by Hawkins et al. in 1990 and is based on the knowledge that SAP is a component of all amyloid deposits and thus when labelled with ^{123}Iodine (^{123}I-SAP) it enables the identification and quantification of visceral amyloid deposits *in vivo*, in a non-invasive, safe, specific and proportionate manner [141, 142]. Although a histological diagnosis remains gold standard, sequential SAP scintigraphy allows disease monitoring over repeated time intervals and an assessment of response to treatment [143]. The ^{123}I-SAP images do not provide a diagnosis of the amyloid subtype but the pattern of involvement seen may make a particular diagnosis more likely i.e. significant bony uptake is characteristic of AL amyloid [144].

The radiotracer used is rapidly catabolised and excreted from the body with the dose of radiation being similar to a lumbar spinal radiograph; it does not accumulate in healthy subjects or those without amyloid [145]. SAP scintigraphy is used in routine clinical practice at the NAC and the 'amyloid load' is interpreted in a semi-quantitative manner as being mild, moderate or heavy for each organ in comparison to the surrounding blood pool. This result has implications for prognosis and also guides management strategies [42, 146, 147].

Although SAP scintigraphy allows imaging of most solid organs and bone, it does not image the skin, nerves, hollow viscus and, importantly, the heart. Additionally, the radiotracer is expensive and not easily available, however it remains, globally, the most efficient modality for assessing amyloid extent and distribution [144]. This investigation was utilised in the studies detailed in Chapters 3, 5 and 6.

Bisphosphonate Bone Tracers

The incidental uptake of 99m-technetium-3,3,-diphosphono-1,2- propanodicarboxylic acid (99mTc-DPD) was first noted in the 1970s and 80s whist being used for bone scanning and this phenomenon was later attributed to cardiac amyloid. The exact mechanism of uptake remains unclear but its use as a sensitive, inexpensive and simple technique in imaging cardiac deposits in ATTR variant and ATTRwt amyloid is now increasingly realised following Perugini et al's findings of 100% sensitivity and specificity of 99mTc-DPD in imaging [148]. Indeed, cardiac amyloid can be identified even if patients are asymptomatic and other imaging modalities do not yet reflect this disease process. These scans are graded using the Perugini Grade 1-3 scoring system: Score 0 - absent cardiac uptake and normal bone uptake. Score 1 - mild cardiac uptake, inferior to bone uptake. Score 2 - moderate cardiac uptake and normal bone uptake. Score 3 - Strong cardiac uptake with attenuated bone uptake.

Notably, tracer uptake occurs in a third of patients with cardiac AL amyloid, although less avidly than in TTR amyloid patients and 99mTc-DPD-SPECT-CT can help to distinguish the two types [149]. In fact, it has been proposed that a high DPD grade, in the absence of haematological markers for an underlying PCD and echo/ CMR imaging suggestive of cardiac amyloid can reliably diagnose TTR amyloid over AL amyloid, without the need for an invasive cardiac biopsy [118].

Biopsy

A biopsy is essential to diagnose amyloidosis and usually, in systemic amyloid, a biopsy of the clinically involved organ is not necessary where a less invasive, cheaper and quicker biopsy, typically is an abdominal subcutaneous fat aspiration, can be taken. Where AL

amyloidosis is suspected, a bone marrow biopsy is taken and this, in conjunction, with a fat biopsy will identify amyloid in 85% of affected patients [150]. If both biopsies do not show amyloid and the index of suspicion remains high, then a biopsy of the affected organ e.g. heart, liver, tongue, skin could be undertaken and an evaluation made, perhaps using the other assessment tools listed such as SAP scintigraphy, as to whether the amyloid is systemic or localised.

The presence of extracellular amorphous eosinophilic deposits on brightfield light microscopy after staining with haematoxylin and eosin may alert the pathologist to the possibility of amyloid [31]. Further demonstration of amyloid deposits demonstrating green birefringence when viewed under cross-polarised light, after staining of tissue with Congo red dye, is the definitive diagnostic test and has been in use since first described by Puchtler et al. in 1962 [3, 4]. Under electron microscopy, amyloid deposits appear as randomly arranged but rigid non-branching fibrils of around 10 nm in diameter and of indeterminate length [5].

Amyloidosis, therefore, is a histological diagnosis either diagnosed following purposeful biopsy of the affected organ or discovered incidentally; amyloid deposits must be followed in all cases by tests to determine the amyloid fibril protein. In clinical practice, this has traditionally been achieved with immunohistochemistry and/or immunofluorescence, although more recently, proteomic analysis of micro dissected amyloid deposits has been used to determine the amyloid fibril protein with great accuracy, as described in Chapter 4. [31]. Sometimes a characteristic distribution of deposits on light microscopy alone can provide useful pointers, for example fibrinogen Aα amyloidosis typically displays isolated heavy glomerular involvement, but generally immune-typing is required to distinguish between the different amyloid subtypes [151]. For example, a diagnosis of AL amyloidosis

should be suspected when there is LC restriction in the glomeruli, interstitium and/or vessels following staining of renal tissue with antibodies against κ and λ immunoglobulin LCs. There are a number of other proteins which can deposit as amyloid in the kidneys such as apolipoprotein A-I (apoAI), apolipoprotein A-II, fibrinogen Aα chain, gelsolin, lysozyme and LECT2 with differing management strategies and therefore identification is essential. DNA sequencing, in addition to immuno-typing is invaluable in diagnosing and excluding the known hereditary forms of amyloidosis.

The importance of biopsies and their evaluation is essential is discussed further in this thesis: in avoiding a misdiagnosis (Chapter 3), in procuring tissue for proteomics (Chapter 4), in correlating with biomarker evaluation (Chapter 5) and identifying Light Chain Deposition Disease (Chapter 7).

Mass Spectrometry and Bioinformatics

Proteomic analysis using mass spectrometry after laser microdissection, is playing an increasing role in diagnosing the amyloid subtype where results from histological and/or genetic tests prove ambiguous [152]. For example, absence of staining in immune-typing may sometimes occur where epitopes are hidden in the protein folds or epitopes are not present in the truncated fibrillary forms of the protein deposits and indeterminate results occur when more than one antibody is stained, perhaps due to plasma protein contamination leading to non-specific background staining [153]. Importantly, proteomic analysis can only be undertaken following histological confirmation of Congo red deposits, as it is this material that is laser micro dissected for mass spectrometry, hence conventional histological identification of amyloid must be performed. This technique has mainly been spearheaded in the United States but is being increasingly used in the National Amyloidosis Centre, UK and its establishment is further described in Chapter 4 [154].

Several mass spectrometry methods are now routinely used for protein identification with standard mass spectrometers readily available in research facilities. Proteomics is dependent on the determination of the mass of ionised protein or peptide i.e. the mass-to-charge ratio (m/z), by the mass spectrometer. The proteolytic digestion of protein, e.g. by trypsin (which cleaves the C-terminal of arginine and lysine, except before proline), generates peptides that are 'charged' by the mass spectrometer and the unique mass of each ionised sequence in highly purified samples can lead to the identification of the peptides present- a process known as Peptide Mass Fingerprinting. For reasons of greater sensitivity, the method of ionisation in most cases is matrix-assisted laser desorption ionisation with the mass analyser being an ion trap device, a subtype of the Quadrupole analyser, which traps selected ions hence allowing greater sensitivity. The other widely used analyser is 'time-of-flight' [155]. Tandem mass spectrometry, MS/MS or tandem MS, is used to further fragment selected peptide ions and the resulting daughter ions are detected in a second mass analyser. The identification is undertaken by 'search engines' which have specific spectral matching algorithms and are reliant upon matching the generated spectra to theoretical spectra generated from a sequence database [156]. Hence, only sequences within the selected database have a chance of being "identified". De novo MS/MS peptide sequencing without the reference to a sequence database can be performed manually by correlating the mass difference between MS/MS peaks, but this is a tedious and understandably unpopular method which would be too time consuming in high throughput diagnostics [157]. Many algorithms have been developed for automated de novo sequencing over the past decade.

Treatment

This is dependent upon the type, severity and distribution of amyloid as well as the clinical status of the patient. Treatments are aimed at reducing the rate of amyloid production whether it be through chemotherapy in AL amyloidosis or the use of anti-inflammatories in AA amyloidosis. Once production decreases, one can occasionally observe regression of existing amyloid deposits- as visualised on imaging and also as evidenced by functional testing i.e. exercise and blood tests [158]. Where such treatment is prohibited by the clinical status of the patient i.e. due to significant co-morbidities, or where either the window of treatment or no suitable generalised treatment is available, then organ specific supportive measures are taken. Importantly these measures may differ to those employed for the management of other conditions e.g. although the general management principles of heart failure are followed including fluid and salt restriction as well as diuretic use, notable differences include the possible avoidance of non-dihydropyridine calcium channel blockers (significant negative inotropy and high degree heart block), digoxin (binds avidly to fibrils and half-life is increased significantly), angiotensin-converting enzyme inhibition and nitrates (may contribute to hypotension) and high dose beta blockade (interferes with demand for a raised heart rate due to a low and fixed stroke volume) [159-162].

AL amyloidosis

Current treatment is centred on suppressing the clonal B cells that give rise to the instigating PCD, and hence reducing the supply of the AL amyloid fibril precursor protein. This may facilitate gradual regression of amyloid deposits and preservation or improvement in vital organ function. There is convincing evidence that more complete clonal responses are associated with both longer treatment free survival and a greater chance of improvement in organ function. In low risk patients, autologous stem cell transplantation is widely regarded

as the treatment of choice [163]. The prognosis of untreated AL patients remains 12-15 months, and just a few months for patients with significant cardiac involvement, highlighting the need for novel, more effective regimes [9].

The agents currently used, including the newer immunomodulatory drugs, as well as the more traditional regimes, are summarised in table 1.5. Current regimes have been modified from multiple myeloma protocols and treatment choice is dependent upon the type and severity of organ involvement for example cardiac involvement, peripheral neuropathy, or significant hypotension may preclude particular agents.

Immunomodulatory Drugs

Thalidomide is most commonly combined with cyclophosphamide and dexamethasone, 33% of patients have been reported to have achieved a complete or very good partial response, after a median of 7 months, but 29% of patients died and 50% of treated patients had to be hospitalised for treatment toxicities mainly: fluid retention, lethargy, infection, hypotension and neuropathy [164, 165].

Lenalidomide has a haematological response rate ranging from 41% to 47% which includes relapsed and thalidomide refractory cases. Doses higher than 15mg are poorly tolerated with side-effects including cytopaenias, fatigue and fluid retention [166, 167]. In a retrospective analysis from the Boston group, 66% of patients exposed to lenalidomide developed renal dysfunction, which was reversible in 44% of cases [168]. However, it was reasonably well tolerated in patients with ESRF. There has also been an observed increase in NT-proBNP and other cardiac biomarkers with the use of immunomodulatory agents and

this has been correlated with mortality [169]. Time to haematological response is longer than that observed with bortezomib, but complete responses have been seen [170].

Pomalidomide

This thalidomide analogue was reported by Dispenzieri et al, to induce a 48% haematological response rate with 3% complete responses in 33 heavily pre-treated patients. One-year progression-free survival and overall survival rates were 59 and 76%. 12 of the 33 patients had renal involvement and 2 of these patients demonstrated organ improvement [171].

Proteosome Inhibitors

Bortezomib induces a rapid decrease in serum free LC concentration in patients with myeloma and purified plasma cells from amyloid patients are twice as vulnerable to bortezomib inhibition as those from myeloma patients [172, 173]. The amyloidogenic plasma cell clone synthesises LCs with a propensity to misfold thus overloading the proteosome and increasing sensitivity to Bortezomib and higher doses of Bortezomib are associated with better haematological responses although side effects also increase. Bortezomib is being increasingly used in those with severe cardiac involvement whose outlook is extremely poor. Venner et al. reported a haematological response of 81.4% using biweekly bortezomib, cyclophosphamide and dexamethasone, superseding that achieved by other combinations including 71% with bortezomib/ dexamethasone, 67% with bortezomib/ melphalan/dexamethasone, and 69% with bortezomib alone [174-176]. Other novel proteasome inhibitors currently undergoing trials include ixazomib and carfilzomib [173, 177, 178].

Family	Primary Agent plus Adjunctive Treatment	Main Side-Effects	Comments
Immunomodulatory	Thalidomide (with cyclophosphamide and dexamethasone	Fluid retention ,fatigue and postural hypotension, peripheral neuropathy, thromboembolism, increase in cardiac biomarkers, skin rash, teratogen	
Immunomodulatory (2nd generation)	Lenalidomide (with dexamethasone)	Fatigue, constipation/diarrhoea, myelosuppression, thromboembolism, skin rashes, increase in cardiac biomarkers, renal dysfunction	Useful in disease refractory to alkylators and bortezomib. Response is not achieved rapidly. Addition of cyclophosphamide or melphalan has improved CR rate but 2/3 patients develop side effects.
Immunomodulatory (3rd generation)	Pomalidomide (with dexamethasone)	Fluid retention, fatigue and postural hypotension, peripheral neuropathy, thromboembolism, myelosuppression,	Useful where myeloma is relapsed/refractory to lenalidomide and thalidomide including cardiac patients

		increase in cardiac biomarkers, skin rash, teratogen	
Alkylator	Melphalan-Dexamethasone	Haematological toxicity, fatigue, peripheral oedema, GI side effects	Good choice for intermediate risk and frail patients without significant cardiac involvement. IV MDex too toxic for routine use but used if poor GI absorption.
Alkylator and Purine Analogue	Bendamustine (and dexamethasone)	Cytopaenias	Useful for relapsed/refractory disease
Proteosome-Inhibitor	Bortezomib (and cyclophosphamide with dexamethasone)	Peripheral neuropathy, hypotension, GI disturbance, peripheral oedema	Advised upfront in those with a poor prognosis where a rapid response is required

Table 1.5 Current chemotherapy regimens for AL amyloidosis

Abbreviations: AL, immunoglobulin light chain amyloidosis; CR, complete response; GI, gastrointestinal; IV MDex, intravenous melphalan-dexamethasone

CPHPC and Anti-Human SAP Antibodies

SAP, a constituent of all amyloid deposits, can comprise up to 14% of the dry mass of amyloid. Its binding serves to both stabilise the amyloid fibrils and protect them from proteolysis and degradation by phagocytic cells [179].

(R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid (CPHPC) is a competitive inhibitor of SAP binding to amyloid fibrils. It consists of 2 D-proline residues joined by a six-carbon aliphatic linker. The palindromic structure of this drug cross-links pairs of SAP molecules in the plasma which triggers their complete clearance by the liver, gradually depleting SAP from the amyloid deposits [124, 180]. It also rapidly depletes SAP from the CSF. Gillmore et al. undertook a prospective study in 31 patients with advanced systemic amyloidosis of AL, AFib, ATTR, Gelsolin, ApoA1, AA and Aβ2M types [181]. The patients received CPHPC by twice daily subcutaneous injection, and six subjects continued to receive CPHPC for another year. This treatment produced a sustained and profound depletion of circulating SAP in all cases, and in the two patients in whom amyloidotic material became available for analysis, there was also substantial SAP depletion from the amyloid deposits. In four of the five dialysis independent patients with AFib amyloidosis, proteinuria decreased whereas proteinuria increased and renal survival was shorter in five of six matched untreated historical controls. Amongst 13 patients with advanced AL amyloidosis, organ function improved in two cases and was stable in seven. In two AL cases with refractory clones and a third with a modest clonal response, organ function deteriorated. The only adverse events attributable to CPHPC were transient minor local stinging at the injection site for two patients. There were 5 ‘expected’ deaths associated with advanced amyloid cardiomyopathy [181]. CPHPC is now being used on compassionate grounds for a select few patients.

The removal of large amounts of visceral amyloid deposits, containing human SAP, in mice was demonstrated following the administration of anti-human-SAP antibodies. This triggered a potent, complement-dependent, macrophage-derived giant cell reaction and no significant adverse effects occurred [23]. Richards et al. have recently reported on the 16-patient clinical trial of CPHPC, to deplete the circulating SAP, prior to the administration of a fully humanised monoclonal IgG1 anti-SAP antibody to activate macrophage destruction of the SAP-containing amyloid deposits in tissues. There were clinically no adverse effects to patients and there was evidence on SAP scintigraphy of reduction in hepatic and renal amyloid loads in patients [7]. However, prognosis in AL amyloidosis especially, is to a large degree determined by the presence and severity of cardiac amyloid and we do not yet know whether anti-SAP antibodies target the heart at higher doses. Further clinical trials are yet to take place.

Device Therapy

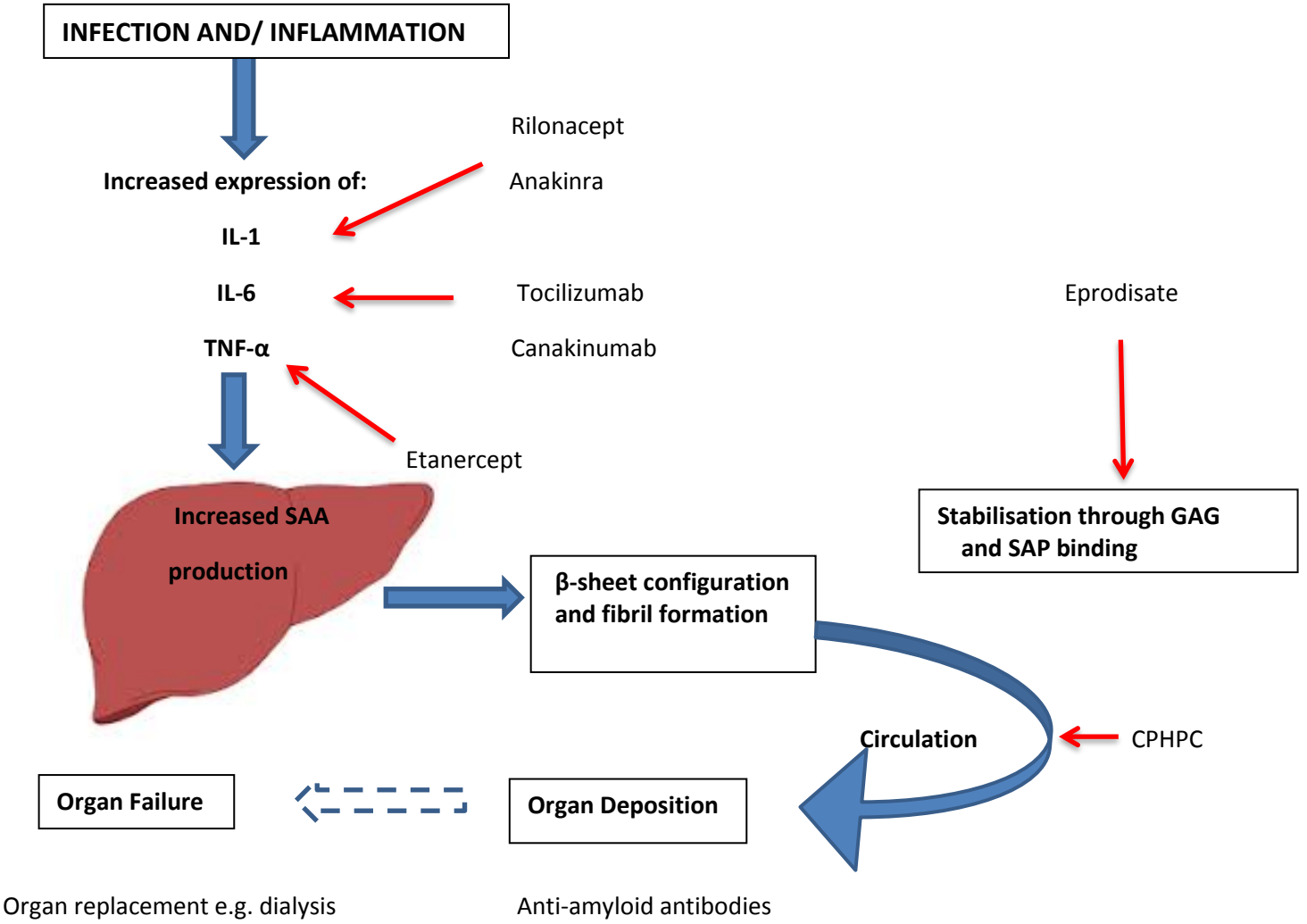
The prognosis of systemic AL amyloidosis is substantially influenced by the presence and severity of cardiac involvement [125]. Greater than two thirds of deaths occurring in patients with AL amyloidosis are secondary to cardiac causes, notably congestive cardiac failure and sudden cardiac death [129]. Despite progress in chemotherapy, sudden cardiac death (SCD) occurs within 8 months in the majority of patients with marked elevations in both TnT and Nt-proBNP and only 29% survive a year [122, 123, 182, 183] . Cardiac death is thought secondary to worsening amyloid infiltration and subsequent chamber hypertrophy and valvular thickening. The severity of cardiac involvement can be evaluated through serum biomarker concentration, echocardiography and CMR [28, 131, 162, 182]. Whilst ventricular tachyarrhythmias and pulseless electrical activity (PEA) have been observed as terminal events, little is known about the electrical activity preceding terminal cardiac decompensation [129, 184, 185]. There has long been interest in, but few systematic studies

of, interventions including anti-arrhythmic drug therapy, implantation of permanent pacemakers (PPM) or cardiac defibrillators (ICD) that might improve survival in cardiac AL amyloidosis [186]. Unfortunately, the very poor prognosis of patients affected by advanced cardiac AL amyloidosis, with a survival from the time of diagnosis reported to be between 5.8 to 7 months, has largely resulted in their exclusion from prospective clinical studies [123, 182]. Furthermore, since consensus guidelines do not support ICD placement for primary prevention of SCD in patients with a life expectancy of less than 1 year; these devices have rarely been implanted in patients with cardiac AL amyloidosis. It is also not entirely clear as to what the exact mode of death is and if it would therefore warrant ICD placement. Studies are conflicting: in one small cohort of patients with advanced symptomatic cardiac AL amyloidosis, ICD insertion did not appear effective in preventing SCD which was attributed to causes 'not amenable to ICD therapy'. In contrast, two recent retrospective analyses of patients with cardiac amyloidosis identified ventricular tachyarrhythmias in a significant proportion of cases, some of which did respond to ICD therapy [187, 188]. In one study, patients receiving ICD therapy survived for an additional 6 weeks to 19 months, however a larger and more recent retrospective study failed to show any survival benefit [189]. It may be that such patients would benefit from higher than normal defibrillator thresholds, due to the massive interstitial expansion of cardiac tissue and that this may be a more effective intervention [189]. Furthermore, although complex ventricular arrhythmias are reportedly common, only couplets have been shown to be an independent predictor of survival and correlate with sudden death [129]. Biventricular pacing appears to have a limited role, theoretically this may be the ideal pacing option to avoid decompensation of the stiffened ventricle as a result of induced dyssynchrony from right ventricular pacing. I have investigated arrhythmias, including the terminal arrhythmias occurring in the worst affected cardiac amyloid patients, through Loop Recorder Insertion. This study is detailed further in Chapter 6.

Systemic AA Amyloidosis

Current treatment has the objective of reducing the production of SAA to healthy normal levels, with the median level in healthy blood donors being 3 mg/L, through control of the respective underlying inflammatory disease. Sustained suppression of SAA production has been shown to result in amyloid regression [42]. Lachmann et al reported an improvement in renal functions in patients with a median SAA concentration of 6 mg/L and deterioration in patients with a median SAA concentration of 28 mg/L [40]. Proteinuria may diminish substantially, albeit gradually over months and years in patients with AA amyloidosis when the underlying inflammatory disease remains quiescent [40]. Essentially, management is aimed at treating the source of inflammation and hence suppressing SAA levels and so, to a degree, treatment is specific to the underlying disorder e.g. surgery for solid tumours, colchicine for Familial Mediterranean Fever and chemotherapy for widespread and/or haematological malignancies. Additionally, more universal therapeutic strategies may be required on occasion to manage acute insults e.g. kidneys extensively infiltrated by amyloid are exquisitely vulnerable to hypoperfusion, hypertension, nephrotoxic drug usage, surgery etc. all of which should be avoided if possible. An overview of novel treatments and their corresponding targets in SAA amyloidosis is shown in Fig. 1.1 below.

. **Figure 1.1 Pathogenesis and novel treatment options available in SAA amyloidosis**



Abbreviations: GAG: glycosaminoglycans, IL-1, interleukin 1, IL-6: interleukin 6, SAA: Serum Amyloid A, TNF: Tumour Necrosis Factor

Colchicine

Familial Mediterranean Fever (FMF), first identified in 1949, is an autosomal recessive inherited condition ethnically restricted to populations, such as Armenians, Turks, Middle-Eastern and Sephardic Jews, arising from the Mediterranean basin and Middle-East. The single amino-acid mutations arise in the Mediterranean Fever gene on chromosome 16p13:3, which codes for the marenostrin/ pyrin protein and can be identified through genetic analysis, with 30% of those with the clinical syndrome having one mutated allele only [190-192]. It is the most common autoinflammatory disease worldwide, believed to arise from pyrin's role in the NLRP3 inflammasome complex leading to increased interleukin-1 β production, resulting in inflammatory attacks [193]. These first occur in childhood and consist of 24 to 72 hour episodes of recurrent fever, severe abdominal pain, arthralgias or monoarthritis, pleurisy, or an erysipeloid rash on one ankle or foot and are characterised by a large tissue influx of polymorphonuclear leucocytes, neutrophilia and a subsequent rapid acute phase response [194]. Subclinical inflammation may exist between attacks.

The alkaloid, colchicine is in widespread use as a life-time long treatment of FMF since 1972 and routine usage has resulted in a 95% reduction in the frequency and severity of attacks and the stabilisation of proteinuria in those patients with established amyloid nephropathy [195-197]. It can be used both acutely and for long term complications including AA amyloidosis. 60% of untreated FMF patients developed nephrotic syndrome secondary to AA amyloidosis resulting in a high mortality rate. There is no significant effect on survival if amyloidosis is absent [198].

Colchicine, interferes with the process of microtubule self-assembly by forming tubulin-colchicine complexes, reduces TNF-α production and interferes with neutrophils binding to the adhesion molecules on the vascular endothelium [199, 200]. At higher doses, colchicine

interferes with L-selectin so impeding leucocyte movement along the vascular endothelium, suppresses phospholipase A2 activation, lysosomal enzyme release and phagocytosis at the transcriptional level [201-203]. The typical colchicine dose is 1.5 to 2mg a day and can be divided if adverse effects occur e.g. diarrhoea and/or abdominal pain [204, 205]. Predisposed patients e.g. those with renal and/or hepatic failure may also suffer from myopathy, leukopenia and neuropathy in predisposed patients (renal and/or hepatic failure) [206]. 5-10% of patients have primary or acquired colchicine resistance [207, 208] . Of note most 'resistant' patients are actually non-compliant. Resistance is believed to arise due to the inadequate activity of the p-glycoprotein pump in granulocytes or due to competitive inhibition of colchicine metabolism via the CYP 450 system. Parenteral colchicine has had some success in patients resistant or intolerant to oral colchicine, perhaps by overcoming the lymphocyte absorption failure in some non-responders [209]. However, the risk of colchicine poisoning is much higher with parenteral usage and can, rarely, result in multi-organ failure. Other more recent options, where colchicine is ineffective or not tolerated, include anti-TNF-α preparations, interferon-α and thalidomide.

Eprodisate

Glycosaminoglycans, such as heparin sulphate, can promote fibril assembly by acting as chaperones during early stages of protein refolding and amyloid formation. Eprodisate (Kiacta, Neurochem) is a negatively charged, sulfonated molecule that is structurally similar to heparin sulphate and works by the competitive inhibition of the interaction between SAA and glycosaminoglycans. It has been found to inhibit the development of AA amyloid in experimental mouse models. A multicentre randomised double-blind placebo-controlled trial in 180 patients with AA amyloidosis-associated nephropathy has been completed [210]. As eprodisate is renally cleared, the dosage of daily drug prescribed was dependent upon creatinine clearance. Study medication was continued for 24 months unless the patient

progressed to ESRD, had a significant adverse event, withdrew from the study or required rescue medication such as cytotoxic agents, colchicine or anti-TNF agents. Rheumatoid arthritis (49% of the patients) and FMF (19%) were the most common underlying inflammatory diseases. Treatment with eprodisate was associated with a 42% reduction in the risk of worsening renal disease (as measured by creatinine clearance) or death (0.37-0.93 P=0.02). More specifically, compared to placebo, eprodisate significantly reduced the: risk of doubling the serum creatinine, risk of a 50% reduction in creatinine clearance and slope of decline in creatinine clearance. In particular, the decline in creatinine clearance was 4.7ml per minute per 1.73m^2 per year greater in the placebo group than in the eprosidate group, a relative difference of 30% [210]. Perhaps surprisingly, there was no significant difference in terms of overall changes in proteinuria. Despite these encouraging results there was concern that some of the apparent drug benefit reflected a poor outcome in the subgroup of placebo patients who had nephrotic range proteinuria at baseline and the US and European regulatory bodies requested a confirmatory second Phase III trial which is ongoing [211].

Table 1.6: Current treatment strategies for AA amyloidosis

Treatment	Description	Studies in SAA Amyloidosis	Notes
Etanercept	Recombinant human TNFR (p75)-Fc fusion protein: comprising two receptors linked by an IgG_1 Fc fragment	TRAPS-reduced symptoms and inflammatory markers [212] RA- improved eGFR, albumin and acute phase reactants as compared to cyclophosphamide [213]	Poor long term adherence Does not completely normalise symptoms or acute-phase reactants [212] Etanercept-resistance Decreased responsiveness with time [214]
Anakinra	IL-1 receptor antagonist	FMF- decrease in acute phase reactants [215] [216] Useful in FMF with spondylitis	Requires a daily injection Used safely in haemodialysis and transplant patient [217]
Rilonacept	IL-1 decoy receptor: extracellular domain of humanized IL-1 type I receptor and the IL-1 receptor accessory protein fused with the Fc portion of IgG_1	FMF- Reduced frequency of attacks by 76% as compared to placebo, in colchicine intolerant patients over 3 months. Improvement in quality of life but duration of attacks not decreased [218] CAPS- Clinical improvement and SAA normalisation [219]	Once-weekly administration Binds both IL-1α and IL-1β with high affinity, and so it has been suggested that rilonacept might have better inhibitory effect in vivo compared to other IL-1 blockers.

			No serious adverse events. Increased incidence of arthralgia, injection site reactions and URTI [219]
Canakinumab	Humanised IgG_1k monoclonal antibody against IL-1β	RA-Improved function, QoL and arthralgia over and above ongoing methotrexate [220] CAPS-improvement in symptoms, eGFR, proteinuria, QoL and SAA levels [221] [222] Gouty arthritis- Improved QoL, symptoms and fewer flares [223] MWS- improved symptoms and SAA levels [224] Systemic-onset juvenile idiopathic arthritis	FDA and EMEA approved for CAPS, familial cold auto-inflammatory syndrome and Muckle-Wells syndrome. Once monthly administration No significant/ life-threatening adverse effects although increased incidence of infections [222]
Tociluzimab	Humanised anti-IL-6 receptor antibody	Stronger than TNF inhibitors Decrease in SAA and proteinuria level [225]	Complex formation of IL-6 with STAT-3 NF-kappaBp65 and p300 is essential for the synergistic induction of the SAA gene and so for SAA transcription [25–26]. Inhibition of IL-6 activity by

			Toclizumab in RA, completely suppresses STAT-3 activity and the subsequent expression of SAA mRNA
Eprodisate	Low molecular weight, negatively charged, sulfonated molecule: Disrupts the heparan sulfate-β-peptide fibril aggregate by competitively binding to the glycosaminoglycan-binding sites, thus inhibiting fibril polymerisation [226]	Eprodisate reduces deterioration of renal function in patients with AA compared to placebo, independently from SAA concentration [210] The confirmatory Phase III trial has finished recruiting in over 20 centers worldwide (clinicaltrials.gov identifier: NCT01215747).	Principally renally excreted so dose adjustment required in renal impairment. Good bioavailability when administered orally and is not protein-bound.

Transthyretin (ATTR) Amyloidosis

Diflunisal

Diflunisal is a non-steroidal anti-inflammatory drug which stabilises tetrameric TTR in vitro by binding via the thyroid hormone receptor sites [227, 228]. Berk and colleagues have recently completed an international, multi-centre, placebo-controlled study of 130 patients with FAP, with neurological outcome as the primary endpoint [229]. The exclusion criteria included patients >75 year old, a history of gastrointestinal bleeding, eGFR <30 ml/min and NYHA Class IV heart failure. Sixty-three patients with a median age of 59.7 years of whom 54.6% had TTR Met30 completed the study, having taken either 250mg diflunisal or placebo daily over a 2 year period and the authors reported a significant reduction in the rate of progression of neurological impairment and an improved quality of life in the diflunisal treated group with 29.7% of the diflunisal and 9.4% of the placebo group displaying a stable neurological score as assessed by the Neuropathy Impairment Score plus 7 nerve tests (NIS+7) at 2 years. Drug related adverse events including gastrointestinal bleeding and renal dysfunction did not differ between the two groups [229].

Tafamadis

Tafamadis, 2-(3,5-dichloro-phenyl)-benzox-azole-6-carboxylic acid), is an orally administered drug that also stabilises the TTR tetramer through its affinity for the T4-binding site, which does not carry the risks associated with nonsteroidal anti-inflammatory drug use [230]. It is protein bound, metabolically stable and ≥94% is excreted unchanged from the digestive tract. Coelho et al., undertook a randomised Phase II/III double-blinded trial that assigned either 20mg oral tafamadis or placebo to 128 FAP patients (mean age 39 years) with the V30M TTR mutation and early neuropathy for a period of 18 months [231]. Plasma

TTR stabilisation was seen in 98% of tafamadis treated patients and none of the placebo arm. The study did not show a significant change in the predefined primary end point but suggested a slowed deterioration in small fibre neuropathy in the tafamadis arm at 18 months with 45.3% of the evaluable diflunisal and 29.5% of the placebo group displaying a stable neurological score by Neuropathy Impairment Score of the Lower Limb (NIS-LL) at 18 months. The most common side-effects included urinary tract infections and diarrhoea. There has been no evidence that Tafamadis affects thyroid function tests however routine monitoring is advised. Of note Coelho's study excluded patients with an eGFR <30 ml/min and there currently remains no evidence of efficacy in cardiac ATTR amyloidosis. This study has split the drug regulatory authorities with European Medicine Agency granting approval for its use in FAP for TTR Met 30 in patients with early disease who can still walk independently. It has not yet been approved by the US Food and Drug Administration.
Ongoing questions relating to whether either of these agents will be beneficial in cardiac amyloidosis are being addressed at present.

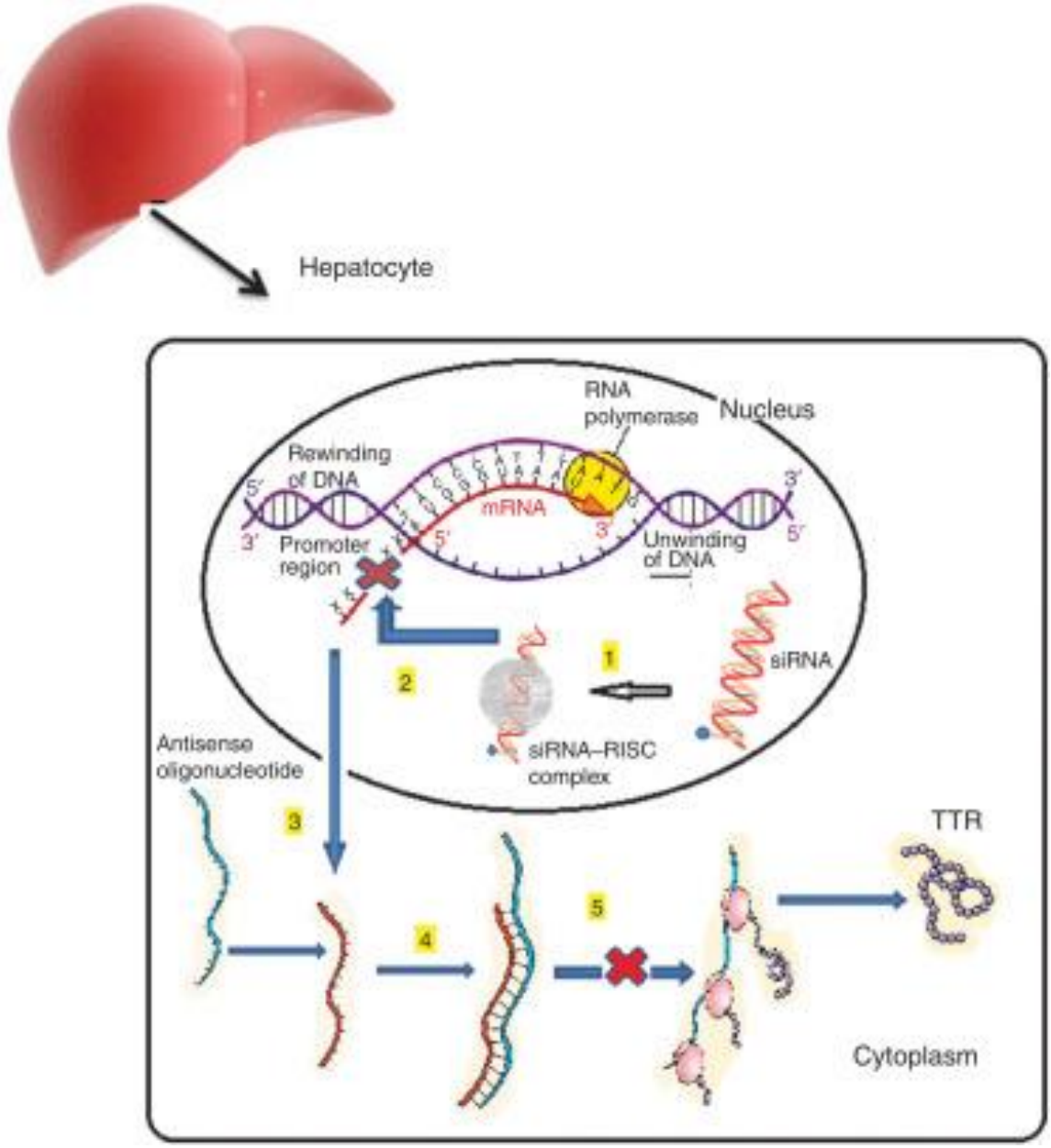
Oligonucleotide Based Therapies

The discovery of RNA interference (RNAi) by Fire and Mello, for which they were awarded the Nobel Prize in Physiology in 2006, demonstrated how the transfer of genetic information from DNA to protein can be blocked [232]. Oligonucleotide based therapies, including small interfering RNAs (siRNAs) and antisense RNAs have the ability to cause changes at the translational level without becoming integrated into the human genome [233].

SiRNAs are non-coding, double-stranded molecules which are components of the endogenous RNAi pathway, which serves to control gene expression. They vary in length from 18-30 base pairs and are chemically modified for drug delivery to increase stability and limit immunogenicity [234, 235]. Lipid nanoparticles have been used to deliver siRNAs to

hepatocytes parenterally; these have resulted in a robust and durable reduction in genetic expression across a variety of species. Antisense oligonucleotides (ASOs) are 13-25 nucleotide single-stranded DNA molecules that hybridise to a specific mRNA sequence and prevent transcription [236, 237]. They can act through an RNase H mediated mechanism, with RNase H being a ubiquitous enzyme that hydrolyses the RNA strand of the RNA/DNA duplex and hence reduces target RNA expression thus inhibiting protein expression to virtually any region of the mRNA. The mechanisms through which both types of oligonucleotide work, is illustrated in Fig.1.2.

Fig 1.2 The mechanism by which small interfering RNAs (siRNAs) block and antisense oligonucleotides disrupt TTR formation



Hepatocyte schematic demonstrating the mechanism by which small interfering RNAs (siRNAs) block the transcription process and antisense oligonucleotides interfere with the translation process, ultimately preventing transthyretin (TTR) formation. (1) The siRNAs bind

to the RNA-inducing silencing complex (RISC) in an adenosine triphosphate (ATP)–dependent manner. (2) This multisubunit protein complex migrates toward messenger RNA (mRNA). At some point, the siRNA unwinds and the antisense strand remains bound to the RISC and blocks transcription by the direct degradation of the target mRNA sequence through the use of both endo- and exonucleases [227, 238]. (3) The mRNA migrates into the cytoplasm where (4) hybridisation with the antisense oligonucleotide prevents protein translocation (5).

Oligonucleotide therapies for ATTR have shown great promise in pre-clinical models, and both siRNAs and antisense oligonucleotides are being investigated in clinical studies (ClinicalTrial.gov NCT01960348 and NCT01737398). Coelho et al., undertook a single-dose, randomised, placebo controlled phase I trial using ALN-TTR01 and ALN-TTR02 (first and second generation lipid nanoparticles), these contained an identical siRNA that bound to an mRNA segment common to both wt and mutant TTR. There was a rapid, dose-dependent and durable reduction of transthyretin levels in the 32 patients with TTR amyloidosis [239]. Alnylam is developing an RNAi therapeutic which has been chemically modified by conjugation to an N-acetylgalactosamine moiety thereby refining hepatocyte targeting and allowing subcutaneous administration, and which is currently in Phase II trial for cardiac ATTR amyloidosis [234].

ASOs have already been tested clinically in the treatment of viral diseases, cancer and metabolic diseases [240-242]. ISIS-TTRRX is a second-generation chimeric antisense inhibitor of its molecular target TTR. It binds selectively and with high-affinity to the non-translated portion of the human TTR mRNA and results in its degradation, preventing production of both wild type and variant TTR protein. ISIS-TTRRX twice weekly sc injections have been well tolerated in both TTR transgenic mouse models and monkeys with a

reduction in hepatic TTR mRNA and plasma wt TTR protein levels by approximately 80% and is currently under evaluation in a phase I clinical trial in normal healthy volunteers [243]. It will initially be developed for patients with FAP and a randomised, double-blind, placebo-controlled study to assess the long-term safety and efficacy of ISIS-TTRRx in patients with FAP was initiated in 2013. The mechanism by which ASOs mediate their effect could be beneficial in other types of amyloidosis. Kluve-Beckerman et al. used ASOs to suppress SAA production in mice and demonstrated that SAA levels in ASO-treated mice were 63% lower than those in controls resulting in reduced AA amyloid deposition [64, 244].

Renal Transplantation

There is some hesitancy surrounding renal transplants in amyloidosis patients, mainly due to fears of recurrent disease and early allograft loss as well as the effect of immunosuppressive regimens on the underlying disease i.e. PCDs in AL amyloidosis and infection/ neoplasm in AA amyloidosis.

The rate of renal allograft decline mirrors that of the native kidneys, in that decline is slower in Lys and AApoA1 where median allograft survival is 13.1 years, as compared to AL, AA and AFib where native decline is typically more rapid and median allograft survival is 8.3 years [245].

From 25 AL patients, there were no graft failures secondary to recurrent amyloid despite amyloid recurrence in 28% of allografts a median of 5.9 years post-transplant and 64% of patients not actually having achieved a complete response to chemotherapy [245]. Amyloid recurrence in AL amyloidosis was not seen where there was complete precursor protein suppression [245]. However, graft survival was significantly worse in those who had not achieved any form of response: 5.3 years vs 8.9 years in those who had responded to

chemotherapy [245]. The median estimated graft survival in AA amyloidosis was 10.3 to 19.3 years, without any significant difference to graft survival in chronic glomerulonephritis [246]. 14 to 20% of patients have recurrent amyloid diagnosed between 5.3 to 9.8 years post-transplant and these patients had significantly higher SAA levels as compared to patients who did not have amyloid recurrence [245, 247]. 5% of grafts failed due to recurrent amyloid [245]. Graft survival noncensored for death was 14.5 years in patients with a median SAA value of <10 mg/L, and 7.8 years in those with a median SAA of >10 mg/L [245]. The most common cause of death post- transplant for any amyloid subtype is infection [245, 247, 248].

Transplantation in AFib patients has a 70% chance of recurrence if there are only isolated renal transplants and no recurrence in cases of combined liver and kidney transplantation; however, these isolated renal allografts lasted 7.3 years as opposed to 6.4 years in those with a liver and kidney transplant. Fourteen patients with ApoA1 had a renal transplant and median graft survival was 13.1 years, so significantly better than all other subtypes and also conferring a survival advantage to transplanted patients [245, 249, 250].

Hence renal transplantation should be considered in patients with amyloidosis, with graft recurrence being less when the precursor protein is sufficiently suppressed i.e. at least a partial response in AL and SAA<10mg/L consistently in AA should be pursued. Although graft recurrence is less in AFibA in those patients where combined liver and kidney transplantation was undertaken, one has to balance this against the high perioperative mortality [251, 252].

Aims and Scope of the Thesis

The over-riding theme and aim of this thesis was to investigate, evaluate and improve upon the diagnostic processes employed in ascertaining both the presence of amyloid and the exact subtype. The secondary aim was to identify and evaluate treatment options in a cohort of patients with the worst prognosis in AL amyloidosis and also to evaluate the treatment options and related prognostic implications in a related disorder, that is Light Chain Deposition Disease.

The introduction is a general overview of amyloid and its subtypes as well as our current knowledge base of this field. The materials and methods section then details the current techniques employed in every study and also the methodology involved in the newer techniques used for this research, in particular, the use of: mass spectrometry and proteomics, urinary retinol binding protein assays and implantable loop recorders.

The specific aims and their derivation for each study are briefly given below and expanded upon in the beginning of each results' chapter:

Misdiagnosing Renal Amyloidosis as Minimal Change Disease

Aim: To evaluate current histological practices employed in diagnosing renal amyloidosis and the potential pitfalls that may lead to misdiagnosis.

Current UK histological practices employed in diagnosing amyloidosis have not been previously evaluated. This study investigates the reasons why renal amyloid was misdiagnosed as minimal change disease, a disorder which presents similarly to renal amyloid. In particular it investigates whether or not Congo red staining and/ or electron

microscopy was undertaken. In cases where correct histological staining took place, I discuss why a misdiagnosis occurred and the impact misdiagnosis has had on patient morbidity and mortality. This retrospective study serves to evaluate current histological practices and the pitfalls present that necessitate advancement in diagnosing amyloidosis.

Mass Spectrometry in the Diagnosis of Renal Amyloidosis and its Subtypes

Aim: to investigate whether a method could be established, namely proteomics, by which amyloidogenic variants, specifically Fibrinogen A α-chain, could be identified in a sensitive and specific manner.

Although mass spectrometry has increasingly ventured into the clinical domain in the US, it had not yet been established in the UK National Amyloidosis Centre. I highlight the deficiencies in current techniques, including the labour intensive IHC process and the failure of IHC to identify the correct subtype in 20-25% of cases, due to the absence of staining, secondary to: epitopes being hidden in protein folds or not present in the truncated fibrillary forms of protein deposits, indeterminate staining perhaps due to background contamination, the loss of epitope detectability in the tertiary structure due to protein cross-linking after formalin fixation or the absence of good quality antibodies [67, 152, 253]. I, alongside colleagues at the NAC, sought to establish proteomics as a diagnostic technique at the NAC and I specifically evaluated its success in identifying Fibrinogen A α-chain variants. I detail how laser capture and mass spectrometry is undertaken and the use of search engines, specifically the modification of the existing Uniprot database by appending a variant database which allows the identification of different pathological variants, including from background contaminants. The use of mass spectrometry in conjunction with IHC in amyloidosis, has increased the diagnostic success rate from 76% to 94% [152].

The Usefulness of Urinary Retinol Binding Protein Measurements in Amyloidosis

Aim: To investigate whether urinary retinol binding protein could potentially serve as a novel sensitive and specific biomarker for renal injury across the amyloid subtypes and, furthermore, provide prognostic information.

The current biomarkers, similar to research-based and clinically applied nephrology in general, used in assessing renal amyloidosis are creatinine, including the derived eGFR and proteinuria. However, creatinine is thought to be a 'late' and non-specific marker of disease progression and there has been a search for a more sensitive and specific biomarker [254]. Urinary Retinol Binding Protein is an important and sensitive biomarker for the loss of function of the human proximal renal tubule and, at the time of my study, had only been investigated comprehensively by one other group and that only in AL amyloidosis and without histological correlation [255]. I undertook a prospective cohort study with 81 patients across different amyloid subtypes and correlated urinary RBP findings with: amyloid precursor proteins e.g. light chains, Serum Amyloid A, histological findings, proteinuria, tubular phosphate reabsorption, glycosuria and amyloid burden. This study also investigated whether urinary RBP had a role in prognostication across a range of amyloid subtypes.

Implanted Cardiac Rhythm Recorders in Advanced Cardiac AL Amyloidosis

Aims: To characterise the nature of and disease-related risk factors for, cardiac arrhythmias in patients with severe cardiac AL amyloidosis, by offering the insertion of an implantable loop recorder (ILR) to a series of consecutive patients with newly diagnosed Mayo stage III cardiac AL amyloidosis

To reveal possible new therapeutic strategies in this cohort of patients, who are known to have the worst prognosis in AL amyloidosis

Approximately a third of patients diagnosed with systemic AL amyloidosis present with cardiac failure and marked elevation of NT-proBNP and TnT [123]. Despite major recent advances in chemotherapeutic strategies for treating AL amyloidosis, these patients frequently die of SCD within 12 months of diagnosis and there has been no improvement in early deaths [123, 256]. Over the past 30 years, the question of whether anti- arrhythmic or chemotherapeutic drug therapy, or the implantation of PPMs or ICDs might improve survival in cardiac AL amyloidosis remains definitively unanswered although ventricular arrhythmias and PEA have been observed [129, 184-186].Unfortunately, due to the exceptionally poor prognosis of patients with advanced cardiac AL amyloidosis, they are rarely included in prospective studies and would not ordinarily be candidates for ICD, in accordance with consensus guidelines [257]. By implanting loop recorders in a cohort of 20 patients with the worst affected cardiac amyloid, I sought to determine, for the first time, the nature of arrhythmias after the diagnosis of amyloid, those immediately before the terminal rhythm and the terminal rhythm itself. This study also sought to assess the effect of treatment strategies, such as the effects of certain chemotherapeutic agents and/ or rate limiting medication and the use of cardiac devices.

Outcome of Light Chain Deposition Disease with Chemotherapy

Aim: To report on the clinical presentation, histologic features, molecular basis and outcome among 53 patients with LCDD who were prospectively followed at the UK National Amyloidosis Centre, highlighting the importance of aggressively treating the underlying monoclonal proliferative disease

LCDD is a multi-system disorder and, similar to AL amyloidosis, arises from the deposition of monotypic immunoglobulin LCs in various organs [258]. The diagnosis, treatment and

prognosis in relation to haematological response and treatment in LCDD have never before been comprehensively evaluated in a large cohort of patients and there are no international guidelines on how to treat LCDD. I undertook a retrospective analysis of 53 patients with LCDD, analysing the presentation, renal histology and treatment of these patients including ASCT and renal transplantation. The use of the haematological response criteria applied to AL amyloidosis permitted a grading of the haematological response and a comparison with AL amyloidosis, allowing an evaluation of the effects of the pathological light chains alone as compared to the light chains within the amyloid structure. This strategy also led to an assessment of the relationship between the haematological response achieved and the patient's prognosis.

Chapter 2: Material and Methods

Declaration

The concepts underlying all the studies resulted from discussions between myself and my supervisor, Dr Julian Gillmore. I subsequently designed the studies, identified and recruited patients, collected data and performed all of the statistical analyses using Excel, Graphpad Prism (Version 4) and/or SPSS v20 (IBM SPSS). Individual statistical methods are discussed separately in each results' chapter. Historical patient information was obtained from the NAC database. I obtained data on deaths and follow up reports from the relevant patients' General Practitioners, local hospital teams and the Office for National Statistics. I performed Congo red staining, laser dissection and prepared specimens for mass spectrometry in the Fibrinogen Aα study and ran results through the search engines and analysed the data obtained. Training was provided by Janet Gilbertson and Dr Nigel Rendell from the NAC laboratories and laser microdissection training specifically was provided by Thomas Adejumo at the MRC Clinical Sciences Institute, UCL with interpretation training undertaken at a Wellcome Trust Proteomics and Bioinformatics' Course.

For the Implantable Loop Recorder (ILR) Study, I was trained by Ms Lindsay Ford at Medtronic's, and I subsequently taught patients on how to use the ILR device including setting up and downloading data remotely. I identified suitable patients, examined, recruited them and informed their local teams of the study and their participation. I co-ordinated patient admission to the cardiology unit and informed the on-call cardiology team and technicians of the patients' admission for an ILR device. I then prepared the pre-operative notes and was available post-operatively to educate patients on how to use the device. Patients contacted me at the time of each download on a weekly basis or whenever they experienced worrisome symptoms. I examined these downloads immediately to rule out any life-

threatening arrhythmias and then reviewed the rhythm strips with a consultant cardiologist: Dr Dominic Rogers, Dr Fakhar Khan or Dr Carol Whelan on a weekly basis. For any patients who died, and as per prior consent, I obtained downloads from mortuary staff. Having collated the data, I performed the statistical analysis. Measurement for biochemical and haematological data were performed by the Royal Free Hospital laboratory services. Informed consent from all patients was obtained and documented.

With regards to the Light Chain Deposition Disease Study and the Retinol Binding Protein Study, amyloid distribution was assessed by myself, Janet Gilbertson and an expert consultant at the NAC. Historical histological data, as reviewed by referring hospitals, Janet Gilbertson and the NAC consultants, was used in the Minimal Change Disease Study. All immunostaining was performed by Janet Gilbertson, Nicola Botcher and Karen Boniface at the NAC laboratories. Gene sequencing was performed by Dorota Rowczenio and Hadija Trojer. SAP scintigraphy was undertaken by David Hutt and Stephanie McKnight. I attended a Wellcome Institute course on proteomics and was trained in data analysis post spectrometry by Drs. Graham Taylor and Nigel Randall at the NAC.

Patients

Patients for all studies were referred to and investigated at the National Amyloidosis Centre. Assessment including examination, history, clinic letters, investigations and serology. These were incorporated into the centre's database, which has >20 years' information. Where earlier or later data was required, I obtained this from General Practitioners, local hospital teams and the Office for National Statistics. All patients were treated in accordance with the Declaration of Helsinki and provided informed consent for all studies included in this thesis and publication of data. Separate consent was obtained for interventional procedures,

namely the insertion of the Implantable Loop Recorder Device Approval was obtained from the Royal Free Hospital Research Ethics Committee.

Functional Assessment

The 6- Minute Walk Test (6MWT)

The 6-minute walk test was used as a standardised measure of functional assessment of cardiothoracic capacity [259]. The same protocol was adhered to by two trained clinical technicians [260]. In brief, the distance walked on the same flat, hard surfaced corridor in a period of 6 minutes was noted for each patient with each patient setting their own walking pace and being allowed to vary their speed and rest as required. All patients rested at least 10 minutes prior to the test, wore comfortable walking shoes and used their usual walking aids. The turnaround point was marked by closed double doors. Patients who had suffered a myocardial infarction within the past month or had unstable angina were excluded (none of the patients included in this thesis). Relative contraindications included a resting heart rate of >120 beats per minute, a systolic blood pressure of more than 180 mm Hg, and a diastolic blood pressure of more than 100 mm Hg. Reasons for immediately stopping the 6MWT include the following: (1) chest pain, (2) intolerable dyspnoea, (3) leg cramps, (4) staggering, (5) diaphoresis, and (6) pale or ashen appearance.

A comprehensive protocol and discussion was obtained from https://www.thoracic.org/statements/resources/pfet/sixminute.pdf.

Eastern Co-operative Group (ECOG) Performance Status

Functional evaluation of patients' performance grades was evaluated in the clinic by using the Eastern Co-operative Group (ECOG) performance status. This is a standardised and numbered scale used to assess a patient's functional capacity, in terms of their self-care, ambulation and level of daily activity (table 2.1). It is widely used by researchers to define their study participants and has been derived from as assessment tool used in oncology to determine a patient's tolerability to treatment dosage or in fact to treatment all together [261].

Table 2.1 Eastern Co-operative Group (ECOG) Performance Grades

Grade	Description
0	No restriction in undertaking pre-disease activities. Fully active.
1	Physically strenuous activity not possible but ambulatory and able to undertake light activity e.g. housework
2	Active for >50% of waking hours, independent for all self care only but no additional activities
3	Limited to certain aspects of self care and sedentary or prone for >50% of waking hours
4	Complete disability, dependent for all self care and confined to bed or chair.

** Adapted from: Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity and Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.*

New York Heart Association Classification

This was used to grade the severity of functional limitations imposed by the presence of heart disease, as scored by the same experienced technician.

Table 2.2 New York Heart Association class and description

NYHA Class	Limitations to Physical Activity	Effect of physical activity
I	None	No undue fatigue, dyspnoea or palpitations
II	Slight	Fatigue, palpitations, dyspnoea or angina pectoris
III	Marked	Symptoms with subnormal physical activity
IV	Severe	Symptoms of CHF even at rest and discomfort with any level of physical activity

Adapted from: National Heart Foundation of Australia and the Cardiac Society of Australia and New Zealand (Chronic Heart Failure Guidelines Expert Writing Panel). Guidelines for the prevention, detection and management of chronic heart failure in Australia. Updated October 2011).

Transthoracic Echocardiography

Echocardiography was performed in all patients with two-dimensional and M-mode settings using a GE Vivid E9 system, by 3 echocardiographers who were experienced in imaging patients with cardiac amyloidosis specifically, with > 40 years' experience between them. Views most commonly used were parasternal long axis and apical long axis. Tissue Doppler was performed immediately above the mitral valve annulus at the septal and left ventricular

septal wall. Left ventricular wall thickness, left ventricular diastolic function, left ventricular systolic function and atrial diameter were assessed using defined criteria from the British Society of Echocardiography (http://www.bsecho.org). Left atrial area was measured using criteria defined by the American Society of Echocardiography (http://www.asecho.org).

The parameters looked at in particular and utilised in this thesis, included:
The thickness of the IVSd (the interventricular septum at end diastole), where <1.1 cm is deemed 'normal,' is believed to be thickened in amyloidosis secondary to fibril infiltration and classically precedes a decrease in LVEF, which tends to be a late finding [162]. Current guidelines use an increase in IVSd thickness with a positive biopsy for amyloid, usually from an extra-cardiac site, to make a diagnosis of cardiac involvement [106].

The ratio of peak early diastolic mitral inflow velocity (E) to peak early diastolic mitral annular velocity (e') i.e. the E/e' ratio, is an independent predictor of survival, which represents the diastolic function. A higher E/E′ index indicates greater LV end-diastolic pressure [262].

The global longitudinal strain, assessed using automated speckle-tracing echocardiography, has been found to be more effective than the conventional measurement of ejection fraction in detecting subtle changes in LV function and better at predicting cardiac events and all-cause mortality [263, 264]. This is the change in length per unit length, a sensitive measure for detecting early systolic dysfunction and reflects the longitudinal contraction of the myocardium.

Histology

Congo- red Staining

All unprepared tissue samples were formalin-fixed and paraffin-embedded. Serial sections were stained with haematoxylin and eosin and six to ten µm-thick renal sections were cut (rather than the usual 2 µm). In brief, sections were dewaxed, for 5 minutes, through each bath of xylene, hydrated through graded alcohol, immersed briefly in Mayer's haematoxylin, rinsed or 'blued' under running tap water, immersed for 20 minutes in filtered alkaline alcoholic NaCl solution, drained briefly, stained with alkalinised Congo red as per the method developed by Puchtler et al., dehydrated with increasing concentrations of alcohol, cleared with xylene and then mounted with resinous mounting medium [265]. All such samples along with previously stained slides were viewed under brightfield and under cross-polarised light by two independent observers at the National Amyloidosis Centre to determine the presence and location of amyloid deposits. Positive controls were obtained from a known Congo red positive block validated by laser microdissection and mass-spectrometry based proteomic analysis which was always processed in parallel.

Immunohistochemistry

Following Congo red staining and confirmation of Congo red deposits, the amyloid subtype was determined by immunohistochemical staining. Formalin fixed sections were deparaffinated in xylitol, 3 × 10 minutes and dehydrated in a descendent alcohol series (100%, 90%, 80%, and 70%, 10 minutes each). After 30 minutes of treatment with 100% formic acid, the endogenous peroxidise activity was inhibited by incubation in aqueous 0.3% hydrogen peroxide (H_2O_2) in methanol, for 30 minutes. The sections were rinsed in

phosphate-buffered saline (PBS) containing 0.05% Tween (Calbiochem) to decrease non-specific background staining and enhance reagent spread. Further non-specific tissue binding was abolished by incubating for 30 minutes in normal, non-immune serum from the species providing the secondary antibody (Vector Part of the ImmPRESS Kit). Sections were incubated overnight with primary antisera at 4^{o}C and rinsed with PBS containing 0.05% Tween (Calbiochem) and labelled with secondary antibodies. Sections were washed in PBS and enzyme-antibody bound complexes were visualised using a metal-enhanced 3,3′-Diaminobenzidine tetrahydrochloride (DAB) (Fisher Scientific solution). A panel of anti-human monospecific antibodies reactive with: SAA (Eurodiagnostica, Huntington UK) AL κ, λ, TTR and lysozyme (DAKO Ltd, Denmark House Ely UK), Apolipoprotein AI (Genzyme Diagnostics) and fibrinogen Aα chain (Calbiochem) were used where appropriate. For TTR staining, pre-treatment was performed for enhanced antigen retrieval using by incubating for 10 minutes with 1% sodium periodate, slides were then washed and further incubated for 10 minutes with 0.1% sodium metabisulphate, washed again and incubated for 5 hours at room temperature with 6M Guanadine dissolved in 0.9% NaCl. Immunohistochemically stained sections were counterstained in haematoxylin, 'blued' under running tap water and stained with Congo red.

Laser Microdissection and Mass Spectrometry

TPrior to liquid chromatography/tandem MS (LC/MS/MS) analysis, paraffin wax was removed from the fresh frozen paraffin embedded specimens. Laser capture microdissection was then undertaken using Photoactivated Localisation Microscopy (PALM) (Carl Zeiss, Oberkochen, Germany) (Fig. 2.1) to cut and extract Congo red positive deposits (2.2), which show up as bright red under fluorescent light (Fig. 2.3). Typically, at least 6-7 glomeruli were extracted. The microdissected tissue was catapulted into the adhesive inverted microfuge tube cap placed directly above the sample. The force for catapulting was

produced by a defocused laser pulse that propelled the sample to an awaiting capture device. This contact-free microdissection allows for greater purity of the sample obtained.

Fig 2.1 PALM microscope used to undertake microdissection. This is a super resolution technique that dramatically improves the spatial resolution of the optical microscope (19)

Fig 2.2 Congo red stained glomerular deposits of amyloid, as viewed under the PALM microscope

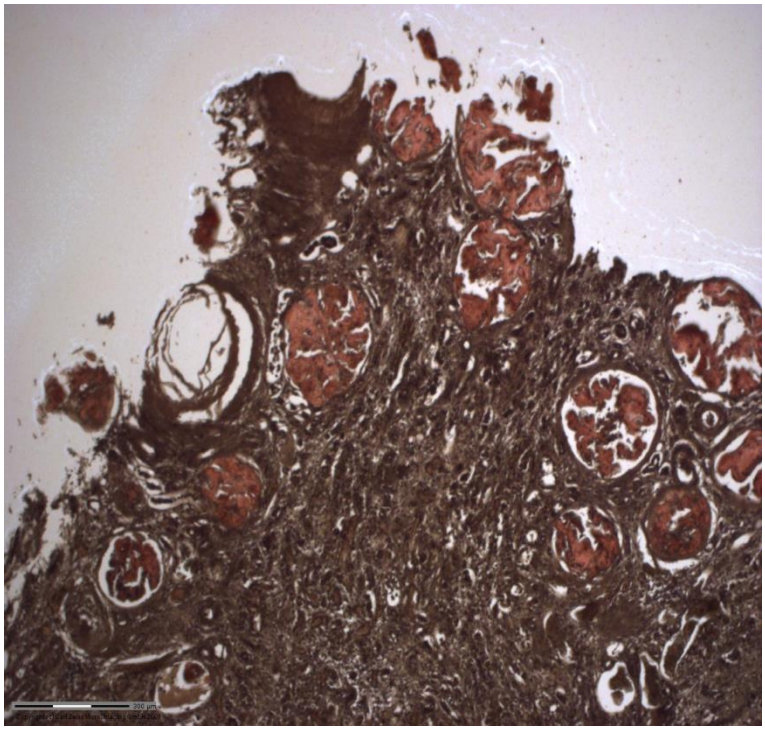
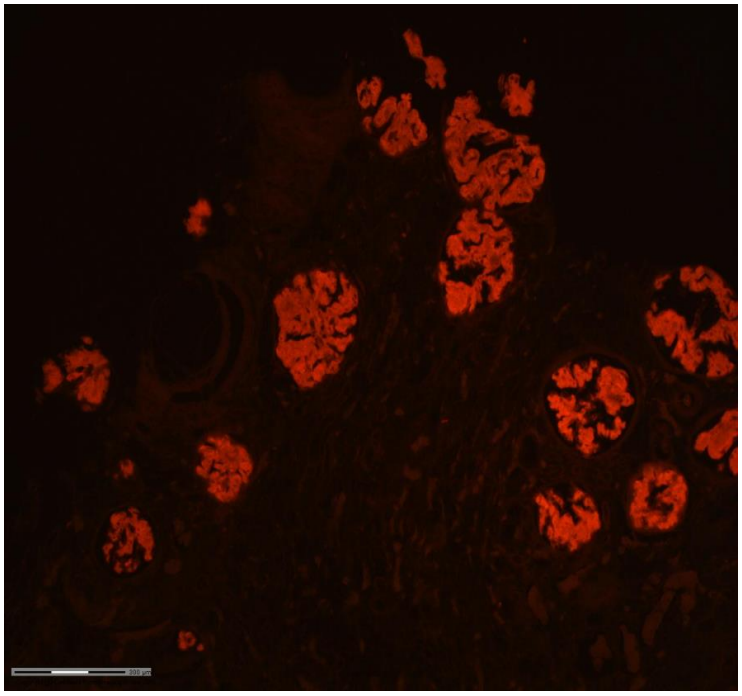


Fig.2.3 Congo red deposits as examined under a PALM microscope, using the tetramethyrhodamine isothiocynate filter, with amyloid deposits appearing red



Sample Preparation for Mass Spectrometry

TEZ (Tris, EDTA and Zwittergent) buffer, composed of the pH buffer Tris 0.1M, the cation chelator disodium EDTA 10 mM and Zwittergent (Z3-16) 0.02% w/v was made up and 0.35 ul was added to each tissue sample. These were then vortexed to enable thorough mixing and left at room temperature for 20 mins. Samples were centrifuged for 5 minutes at 5000 rpm to allow the liquid to settle to the bottom of the eppendorfs. They were then heated for 90 mins at 99°C (Techne Dri-Blok) mixing every 20 to 25 minutes, cooled at room temperature for 5 minutes then centrifuged at 5 minutes for 5000 rpm to recover as much condensate as possible from the tube lids. The eppendorfs were then sonicated. 245 µl of TEZ was added to 5µl of stored frozen trypsin and 1.5 µl of working trypsin was aliquoted into each sample, this was vortexed and centrifuged at 5000 rpm for 1 minute. Samples were digested overnight at 37°C. 5 µl of dithiothreitol (DTT) was placed in each eppendorf to reduce the trypsin generated digests, vortexed and then heated at 99°C for 5 mins and then centrifuged again. This was then frozen at -80 °C for 5 minutes and placed in a vacuum to freeze dry for up to 24 hours. Each freeze dried sample was re-constituted with 20 µL of 1% MeCN/aq and 0.1 % TFA (20 µL/sample) was added to each freeze dried sample and this was sonicated for 5-10 minutes, then spun at 3000 rpm for 5 minutes. 18 µL of this solution was then transferred to the mass spectrometry vials [266, 267].

Protein Identification via Mass Spectrometry

The trypsin generated digests were reduced with 0.1% dithiothreitol and separated by nanoflow liquid chromatography–electrospray tandem mass spectrometry using a Thermo Scientific™ Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Inc.) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA, USA). The MS/MS raw data files were submitted to a workflow tool and the Mascot search algorithm was used. The results were assigned peptide and protein probability

scores. All searches were conducted with variable modifications and restricted to full trypsin-generated peptides allowing for two missed cleavages. Peptide mass search tolerances were set to 10 p.p.m. and fragment mass tolerance to ±1.00 Da. The human SwissProt database was utilised. The MS data show peptides and spectra that match a particular protein based on the amino-acid sequence available in the database. Some of the peptides (and spectra) from different proteins can be common and be shared depending upon the homology of their amino-acid sequence. On the other hand, unique peptides and spectra are distinctive to the particular protein. The ‘Spectra' value indicates the total number of mass spectra collected on the mass spectrometer and matched to the protein using the proteomics software. A higher number of mass spectra is indicative of greater abundance and will typically yield greater amino-acid sequence coverage. A higher mass spectra value also indicates a higher confidence in the protein identification. Amyloid was confirmed by presence of the ‘amyloid signature’ defined as the presence of two or more of the following proteins: apolipoprotein E, apolipoprotein A-IV and SAP. Following the initial search results, four known amyloidogenic variant fibrinogen A α-chain peptide sequences, including the commonest E526V (pGlu545Val) variant, were added to the Uniprot database of the complete human proteome. Samples containing fibrinogen A α-chain were interrogated via the new database.

Serological Tests

Measurement of Retinol Binding Protein

Analysis of urinary RBP was undertaken using a very sensitive immuno-nephelometric technique involving the use of latex enhanced antibodies (the Binding Site Group Limited, Birmingham), according to the manufacturer’s protocol and employing an ELISA technique [268]. A Siemens BN II nephelometer Analyser II was used. This method is dependent on the measurement of light scattered by particles in solution, the particles in this case being

insoluble RBP-antibody immune complexes and the scattered light being directly proportional to the antigen concentration. Prior to RBP analysis, calibration curves were initially prepared using a series of diluted standards of known RBP concentration. Samples of unknown RBP concentration were then assayed and concentration levels determined using the calibration curve. Latex-enhanced antibodies were used to enhance the light-scattering ability of the antibody-RBP complexes and hence avoid the complication of undetection secondary to immune complexes that are too small. This assay was undertaken by Dr Jahn Persaud at the Royal Free Biochemistry laboratory.

Serum Free Immunoglobulin Light Chain Assay

Both κ and λ serum FLCs were measured using a latex-enhanced immunoassay (The Binding Site, Birmingham, UK) on a Behring BNII autoanalyser which uses sheep anti-human polyclonal antibodies that are directed against epitopes which are only revealed when κ or λ light chains are free, as opposed to when they are part of an intact immunoglobulin molecule (Dade Behring, Marburg, Germany). The sensitivity of the assay is <5 mg/L. The reference range was established after having tested sera from 100 healthy blood donors, where the mean concentrations of polyclonal free κ and free λ light chains were 11.38 mg/L (95% CI, 7.41-16.77 mg/L) and 17.36 mg/L (% CI, 8.91-29.87 mg/l) respectively. The normal κ: λ ratio is 0.26 to 1.65 [114, 115]. Because FLCs undergo glomerular filtration, the ratio, rather than the absolute level, is the relevant measurement in individuals with renal impairment. A κ: λ ratio of <0.26 strongly suggests the presence of a population of plasma cells that are producing clonal λ free light chains, whereas a ratio >1.65 suggests production of clonal κ FLCs [269].

In AL amyloidosis, as in LCDD, the production of pathogenic LCs arising from a plasma cell clone is directly responsible for the disease process and halting this production is of

paramount importance. Hence, the involved free LCs, usually κ in AL amyloidosis and λ in LCDD should be measured regularly and the dFLC is calculated. Worsening CKD, in individuals without a proliferative disorder, results in an increase in both FLC isotypes; however, levels of κ LCs selectively increase as they are produced in greater abundance relative to λ light chains and in CKD the difference between the clearance of the smaller monomeric κ LC as opposed to the larger dimeric λ LC is reduced. This has led to the adoption of a revised ratio of 0.37-3.17 in individuals with CKD [270, 271]. Pallidini et al., have devised a grading response system, adapted from that used in myeloma, for AL amyloidosis and demonstrated the correlation of dFLC with survival [272]. This correlation is seen as early as 3 months post treatment. The dFLC was compared across a series of time points for each individual patient, to monitor disease progression including response to treatment, as standardised by Pallidini et al. (Table 2.3).

Table 2.3: Haematologic response criteria for disease caused by light chains

Response	**Criteria**
Complete Response	The normalisation of κ and λ ratio No detectable monoclonal protein by serum and urine immunofixation electrophoresis
Very Good Partial Response	Decrease in dFLC to <40 mg/l
Partial Response	>50% decrease in dFLC
No Response	≤50% decrease in dFLC

Adapted from Palladini et al. 2012 [117]

Gene Sequencing *(as per the method described by Dr Rowczenio)*

Following patient consent, an EDTA tube of whole blood was taken. Gene sequencing was performed on the first visit if a hereditary amyloid subtype was strongly suspected or the sample was stored, should hereditary amyloid be queried at a later date if investigations for the more common subtypes prove futile. Genomic DNA was isolated by a rapid method [273]. The blood was added to NH_4CL and spun, the sample was then re-suspended in 0.9% NaCl and re-spun. It was then suspended again in 0.05M NaOH, incubated, cooled and neutralised with 1M Tris pH8. PCR was undertaken to amplify the fragments, with Ready-To-Go tubes (Amersham Pharmacia Biotech) with the use of solutions and cycling conditions that have been previously described [274]. These were used to amplify the coding regions for: transthyretin (exons 2,3 and 4), apolipoprotein AI (exons 3 and 4) and fibrinogen A α-chain (exon 5) and lysozyme (exon 2). The PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol and automated sequencing of the products was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the procedures recommended by the manufacturer. Gene sequences were analysed on the ABI 3130xl Genetic Analyser using Sequencing Analysis Software version 5.4. The primers used for the PCR process are shown in table 2.4.

Table 2.4 Primers used in the PCR process for genotyping hereditary amyloidosis

Gene (exon)	Forward primer sequence	Reverse primer sequence
Transthyretin (2)	5'-TTTCGCTCCAGATTTCTAATAC-3'	5'-CAGATGATGTGAGCCTCTCTC-3'
Transthyretin (3)	5'-GGTGGGGGTGTATTACTTTGC-3'	5'-TAGGACATTTCTGTGGTACAC-3'
Transthyretin (4)	5'-GGTGGTCAGTCATGTGTGTC-3'	5'-TGGAAGGGACAATAAGGGAAT-3'
Apolipoprotein (3)	5'-GGCAGAGGCAGCAGGTTTCTCAC-3'	5'-CCAGACTGGCCGAGTCCTCACCTA-3'
Apolipoprotein (4)	5'-CACTGCACCTCCGCGGACA-3'	5'-CTTCCCGGTGCTCAGAATAAACGTT-3'
Fibrinogen (5)	5'-AGCTCTGTATCTGGTAGTACT-3'	5'- ATCGGCTTCACTTCCGGC-3'
Lysozyme (2)	5'-GTTATATTGTTCGTTGGTGT-3'	5'-CATTTGTATTGAGTCTCAATTC-3'

SAP Scintigraphy

SAP scintigraphy was undertaken in all patients at their baseline assessment and only repeated at clinic follow-up visits at 6 or 12 months, if clinically indicated. Contra-indications included pregnancy and relative contraindications included claustrophobia. Highly purified SAP was radiolabelled with the medium-energy, short half-life pure gamma emitter ^{123}I and each subject undergoing SAP scintigraphy received approximately 200µg of SAP with

190MBq of ^{123}I by bolus intravenous injection. This is equivalent to 3.8 mSV of radiation and comparable to the dose received for an IV pyelogram. If amyloid is absent then labelled SAP is not retained and is rapidly excreted post catabolism, whereas is individuals with amyloid, the tracer is localised to the affected sites in proportion to the amyloid amount present. Thyroid uptake was blocked by administrating 60mg of potassium iodide immediately prior to the study and then 5 further doses over the next three days. Anterior and posterior imaging was performed at either 6 or 24 hours after injection using an Infinia Hawkeye gamma camera (General Electric). The images were reviewed at the time of acquisition by a panel of physicians with experience of over 29 000 scans. The diagnostic sensitivity in patients with AA amyloidosis is 100% and ~90% in AL amyloidosis.

The scoring of the amyloid load, as standardised by Professor Hawkins at the NAC, was as follows:

Table 2.5: Amyloid load scoring

Total Amyloid Load	**Description**
None	No abnormal tracer localisation. Normal blood pool signal
Small	Tracer uptake into one or more organs with blood pool clearly visible at normal intensity
Moderate	Tracer uptake into organs with blood pool only just visible
Large	Tracer uptake into organs with loss of blood pool signal following adjustment of grey scale to encompass target organ

Regression of the amyloid burden was defined as a reduction in tracer uptake in affected organs or an increase in the blood-pool background signal, whereas an increase in tracer

uptake in affected organs, an abnormal tracer uptake in a previously unaffected organ, or a decrease in the blood-pool background signal suggested progression of the amyloid load.

Implantable Loop Recorder (ILR) Insertion and Data Capture for Cardiac Study

ILRs (Reveal DX or Reveal XT, Medtronic, Minnesota, USA) were inserted subcutaneously under local anaesthetic, using standard implantation techniques, by a cardiologist. Appropriate sensing was confirmed in all cases. Devices were programmed to allow storage of three manual activations (each lasting up to 7.5 minutes); settings for automatic recordings of arrhythmias were as follows: bradycardia - <30 beats per minute (bpm); pauses - >3 seconds; VT - >176 bpm; and fast VT - >231 bpm. Up to 30 episodes of each of those 4 arrhythmia types (bradycardia, pause/asystole, VT, fast VT) were stored per download, with each episode recorded for 60 seconds. Prior to discharge, patients and their next of kin were taught how to activate their device and instructed to perform an activation immediately after any symptomatic episode (palpitations, pre-syncope or syncope) by myself. They were taught how to download recordings using the Carelink remote monitoring system (Medtronic, USA) and received instruction on how to download recordings at weekly intervals in order to capture arrhythmias recorded automatically by the ILR, as well as after any manual activation of the device. Patients contacted me at the time of download and recordings were then reviewed immediately to exclude a malignant arrhythmia. All recordings, including episodes of bradycardia, tachycardia, pauses and atrial arrhythmia, were then interpreted by a cardiologist, either on a routine weekly basis or as soon as possibly if intervention was deemed likely.

Results & Discussion Section

Chapter 3: Misdiagnosing Renal Amyloidosis as Minimal Change Disease

This chapter is based on the publication

Misdiagnosing renal amyloidosis as minimal change disease. Sayed RH, Gilbertson JA, Hutt DF, Lachmann HJ, Hawkins PN, Bass P, Gillmore JD. Nephrol Dial Transplant. 2014 Nov; 29(11):2120-6.

My contribution was undertaking the study and data analysis and subsequently writing the publication.

Aims The aim of this study was to look at current histological practices employed in diagnosing renal amyloidosis and the potential pitfalls that may lead to misdiagnosis.

Introduction

Renal amyloidosis and Minimal Change Disease (MCD) typically present with heavy proteinuria and the nephrotic syndrome (NS). Despite their similar clinical presentations, therapeutic interventions aimed at inducing clinical remission differ substantially between these conditions. Similarly, outcomes differ markedly between patients with these two renal lesions; amyloidosis is usually a multi-system, progressive disease with a poor renal and overall prognosis, whereas MCD is typically a non-scarring, renal-isolated disease which remits following commencement of immunosuppressive therapy and has a good renal and overall prognosis.

MCD accounts for approximately 90% of cases of NS in children who are <10 years old, 50% of cases among children between 10 and 18 years, and 10-15% of adults presenting with NS [275]. Histologically, it is characterised by a lack of any detectable abnormality on light microscopy (LM), other than possible slight mesangial prominence. On electron microscopy (EM) there is epithelial cell foot process effacement. Immunohistochemical staining in MCD may sometimes demonstrate low intensity staining for C3 and IgM but is often negative [276]. Since MCD accounts for 90% of cases of NS in young children and is typically steroid-responsive, the usual practice in those presenting with NS from this age group is to initiate treatment with corticosteroids without recourse to a diagnostic kidney biopsy [277]. In contrast, adults who present with NS usually undergo a diagnostic kidney biopsy before commencement of disease-modifying therapy. Only ~75% of adults with MCD are steroid responsive and there may be a delay of 3-4 months before the response is achieved; total steroid exposure, with tapering, is usually around 6 months [276, 277]. The Kidney Disease Improving Global Outcomes guidelines recommend Calcineurin Inhibitors (CNIs) and cyclophosphamide in those intolerant of high-dose corticosteroids or those who are steroid unresponsive [277]. Renal amyloidosis is rare in children but accounts for 2-5% of cases of NS in young adults, and 11-13% in middle and old age [278].

Here, we highlight a cohort of patients diagnosed with MCD on renal biopsy following a proteinuric clinical presentation, in whom a subsequent diagnosis of renal amyloidosis was established. We investigate the validity of the initial diagnosis of MCD and attempt to establish whether ‘standardised’ operating procedures in the histology laboratory might enable a greater detection rate of renal amyloid deposition.

Methods Specific to this Study *(please see 'Materials and Methods' Section' for more extensive descriptions)*

Study Design and Patients

A retrospective analysis of 2116 patients referred to the UK National Amyloidosis Centre (NAC) with amyloid on renal biopsy between January 2001 and July 2013 was conducted. Those patients in whom an initial diagnosis of MCD had been made were identified through a search of patient referral letters and corroborated in all such cases, by original biopsy reports. This constitutes the study population. Patient demographics at the time of renal amyloid diagnosis, disease-modifying therapeutic interventions, time between the diagnosis of MCD and the diagnosis of renal amyloid, as well as clinical outcomes were evaluated in the study population.

Histology

Kidney biopsy samples (unstained formalin fixed, paraffin embedded blocks and stained slides) and histology reports detailing a diagnosis of MCD (from the study population) were obtained. Six µm thick renal sections were cut and stained with alkalinised Congo red by the method developed by Puchtler, Sweat and Levine in 1962 (Fig. 3.1a) [265]. In each case that amyloid was present, identification of the fibril protein type was sought using a standard panel of antibodies (Fig. 3.1b). All such samples along with previously stained slides were viewed under cross polarised light by two independent observers at the NAC to determine the presence of renal amyloid deposits that initially had been missed (Fig. 3.1c). Wherever possible, EM reports were obtained and/or EM pictures were reviewed or EM was retrospectively undertaken on the relevant biopsy samples in order to identify the presence or absence of fibrils within the kidney tissue (Fig. 3.1d).

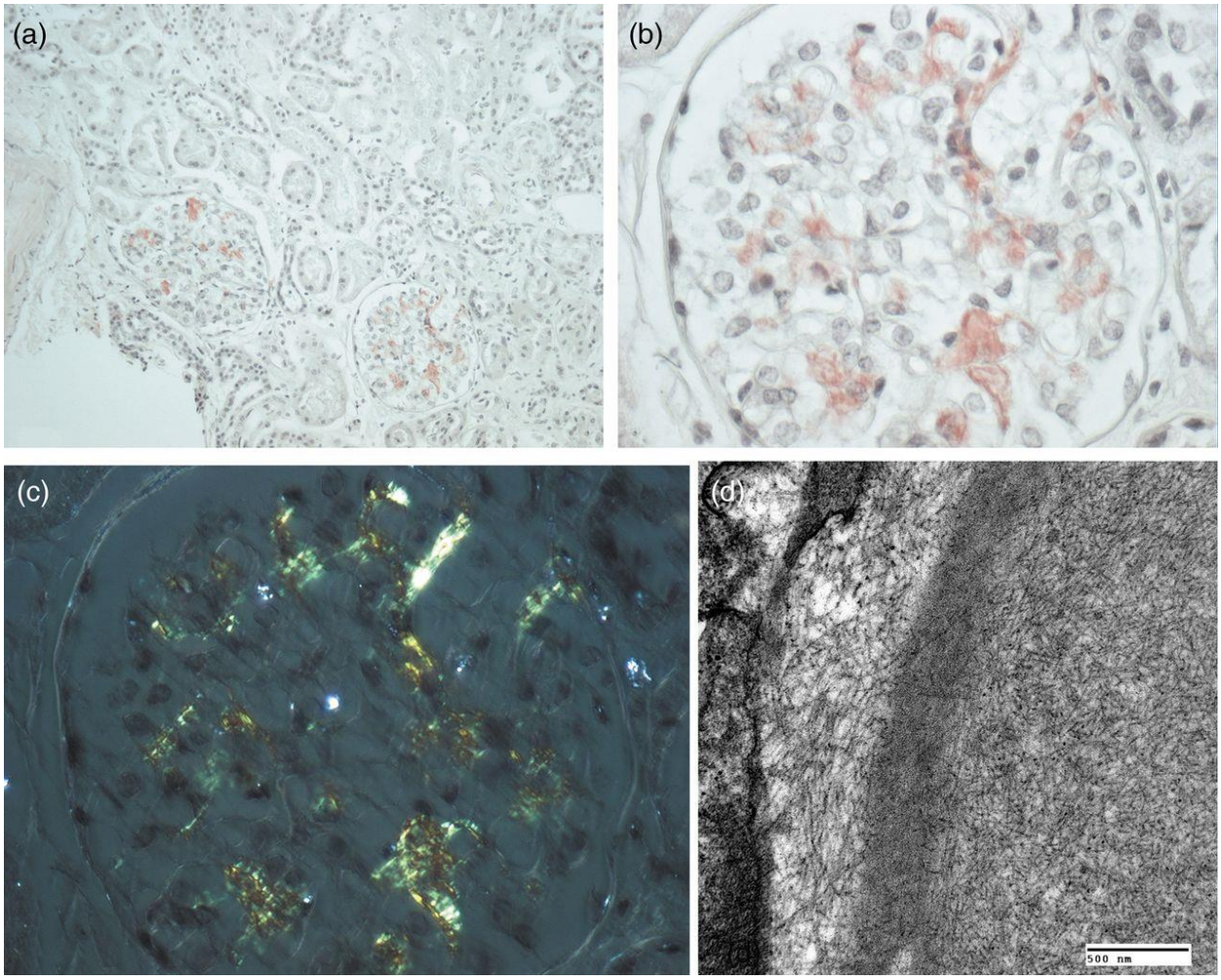


Fig 3.1 Renal histology showing amorphous material within the glomeruli staining with Congo Red at ×100 magnification (a), and a single glomerulus with Congo red deposits at ×400 magnification (b), apple green birefringence under cross-polarized light after staining with Congo red in the same glomerulus at ×400 magnification (c) and EM showing randomly orientated fibrils (d).

Radiolabelled ^{123}I-SAP scintigraphy

SAP scintigraphy was performed during the initial evaluation at the NAC, a median 21 days after histological confirmation of renal amyloidosis. Individual organ involvement by amyloid and whole-body amyloid burden were recorded in each case, as previously described (Table 2.5).

Results

Twenty-seven patients were identified in whom the initial renal histology was reported as MCD prior to subsequent identification of renal amyloid. Patient characteristics at the time of review at the National Amyloidosis Centre, shortly after the diagnosis of amyloidosis, are detailed in table 3.1. Thirteen patients were female and median age at time of diagnosis of MCD was 62 years (range 32 to 73 years). The median time lapse between the diagnosis of MCD and that of renal amyloidosis was 241 days and the range was from 20 to 3632 days. The amyloid type was AL in 25 cases and AA in 2 cases.

By the time of review at the NAC, median proteinuria was 6.9 g/24 hours (range 0.3-12.2 g) and median eGFR was 64 ml/min (range 6 to 191 ml/min). Extra-renal amyloid deposits were identified by SAP scintigraphy (Fig. 2) and/or echocardiography in 21/27 (78%) cases at the initial NAC evaluation and 9 (33%) patients had a moderate or large total body amyloid burden by SAP scintigraphic criteria at this time (Table 1, Fig 3.2 and 3.3) [279]. Ten of 25 (40%) patients with AL amyloidosis had significant cardiac infiltration and both patients with AA amyloidosis had liver involvement, poor prognostic features in the respective amyloid types [125, 146].

Table 3.1: Patient characteristics at the time of initial review at the NAC

Patient	Gender M/F	Age yrs	Amyloid Subtype	Presenting eGFR ml/min/1.73m^2	Presenting Proteinuria g/24 hr	Presenting Amyloid Load on SAP Scan	Extra-Renal Organ Involvement at time of Diagnosis of Amyloid	Renal Replacement Required?
1	F	38	AL	96	5.99	Large	Liver, spleen, heart	No
2	M	73	AL	12	10.7	Small	Spleen	Yes
3	M	70	AL	91	4	Small	Spleen, heart	No
4	F	63	AL	29	7.31	Small	Spleen	Yes
5	F	66	AL	172	10.67	Equivocal		No
6	M	63	AA	16	8.07	Moderate	Liver, spleen	Yes
7	M	61	AL	185	5.4	Moderate	Liver, spleen, heart	No
8	F	61	AL	116	6.72	Large	Liver, heart	No
9	F	53	AL	122	7.5	Equivocal		No
10	F	50	AL	20	4.12	None	Heart	No
11	F	64	AL	56	3.4	Small	Spleen, heart	No
12	M	72	AL	6	5.83	Small	Spleen	Yes
13	M	62	AL	45	6.9	Large	Liver, spleen	Yes

14	F	66	AL	64	11.57	Small	Spleen	No
15	M	61	AL	11	0.29	Moderate	Spleen, liver	Yes
16	F	41	AL	131	5.1	Small	Liver, spleen, heart	No
17	M	51	AL	128	5.5	Small		No
18	M	80	AL	90	3.54	Small	Spleen, heart	No
19	M	70	AL	57	9.1	Small		No
20	M	32	AA	191	8.8	Moderate	Liver, spleen	No
21	M	67	AL	51	9.17	Small		No
22	F	41	AL	67	11.6	Small	Heart	No
23	M	69	AL	47	7.8	Small	Spleen	No
24	F	53	AL	21	6.3	Small	Heart	Yes
25	M	46	AL	107	9.89	None		No
26	F	68	AL	93	4.25	Moderate	Spleen	No
27	F	40	AL	43	12.23	Moderate	Spleen	No

Fig 3.2 Extra-renal amyloid infiltration of organs at the time of presentation to the NAC

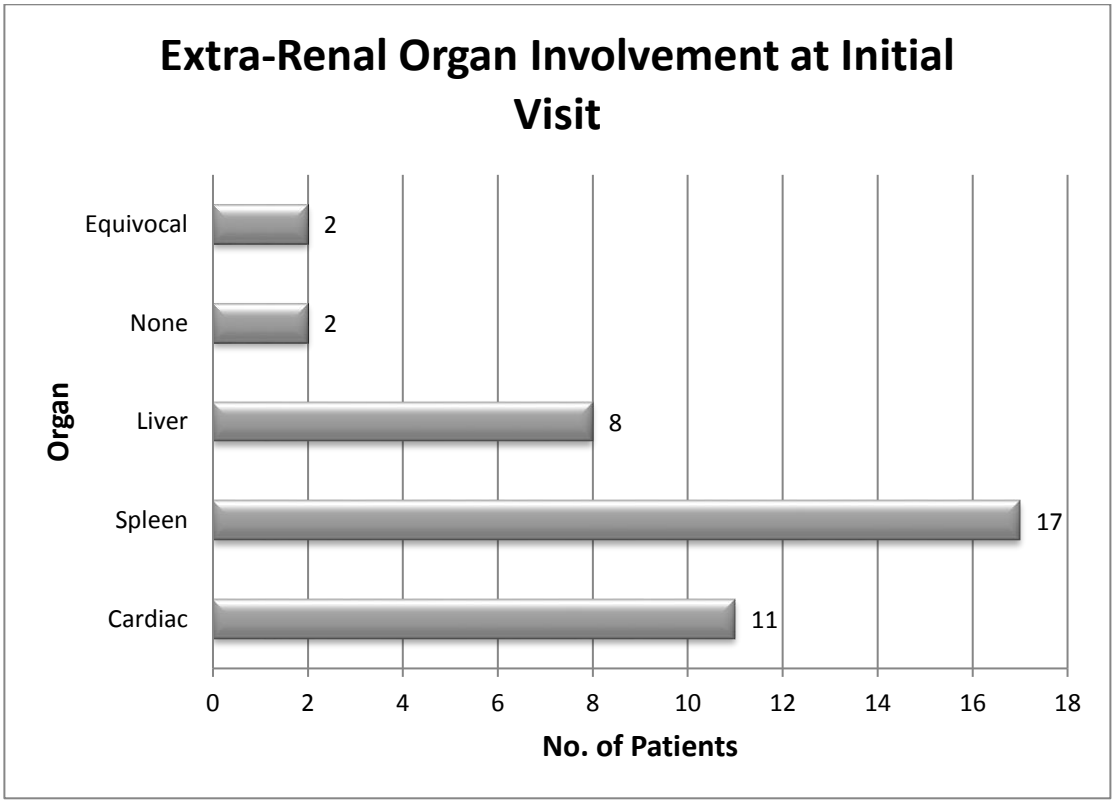
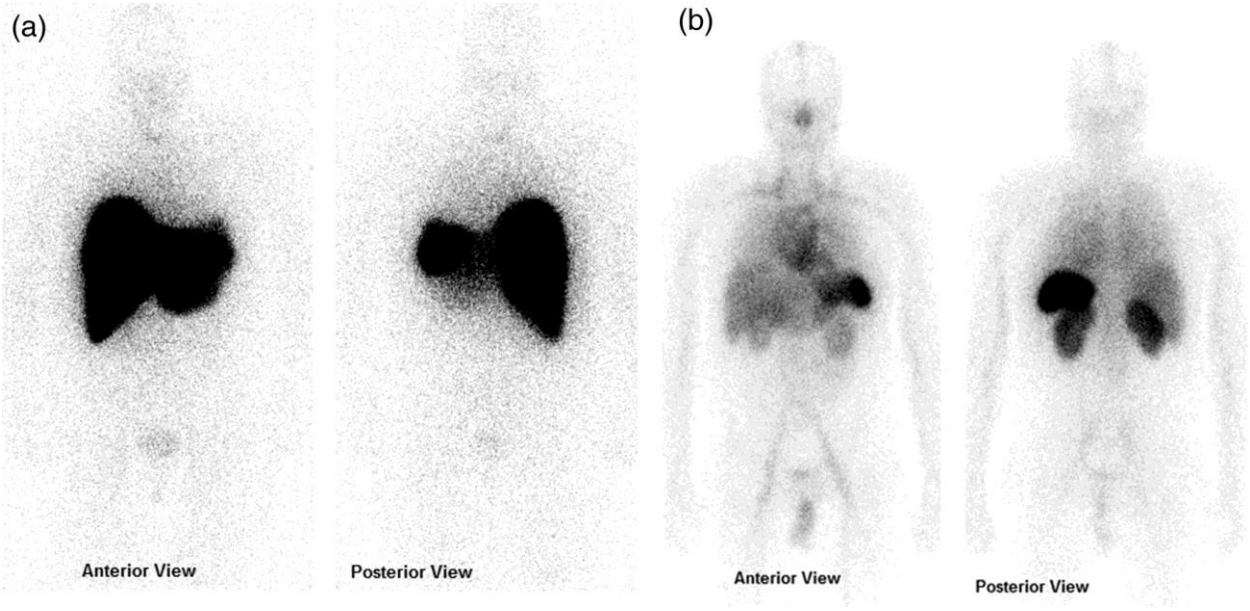
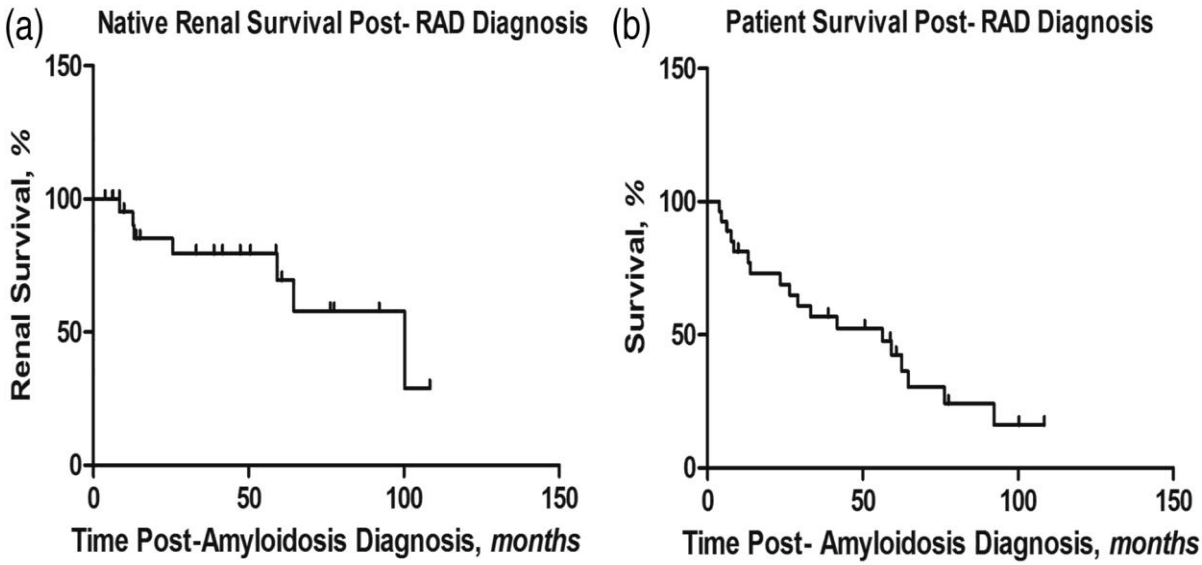


Fig. 3.3: (a) Anterior and posterior whole body SAP scans showing a large total body amyloid load, with tracer uptake in the liver and spleen in a case of systemic AL amyloidosis. The kidneys are obscured by the large burden of amyloid in those organs. (b) Anterior and posterior whole body SAP scans showing amyloid in the spleen and kidneys of a patient with systemic AA amyloidosis



With median follow up in the whole cohort of 3.3 years, 18/27 (67%) patients died, and 7/27 (26%) patients required dialysis (including 2 who were dialysis dependent at the time of diagnosis of renal amyloidosis). Median estimated time to dialysis, from the time of the initial MCD diagnosis was 8.6 years and from diagnosis of renal amyloidosis was 8.3 years (Fig 3.4a). Median patient survival by Kaplan Meier analysis was 5.2 years (range 0.8 to 8.4) from diagnosis of MCD and 4.7 years (range 0.3 to 6.4) from diagnosis of renal amyloid (Fig 3.4b).

Fig 3.4: Kaplan–Meier survival analyses. (a) Renal survival from the time of diagnosis of renal amyloid. (b) Patient survival from the time of diagnosis of renal amyloid.



All subsequent analyses were carried out on 26 of the 27 patients since neither the initial biopsy nor the report for one patient could be retrieved. Seventeen of the 26 (65%) patients were diagnosed with MCD without Congo red staining of kidney biopsies having been performed (Fig 3.5a). All 17 biopsies did however contain amyloid deposits that were evident when sections were stained with Congo red by the method of Puchtler *et al* and viewed under cross polarised light at the NAC [265]. Interestingly, 1 of these 17 biopsies had been stained with Sirius Red at the local hospital, and was reported as equivocal for amyloid despite a 'final diagnostic' report indicating MCD (Fig. 3.5a).

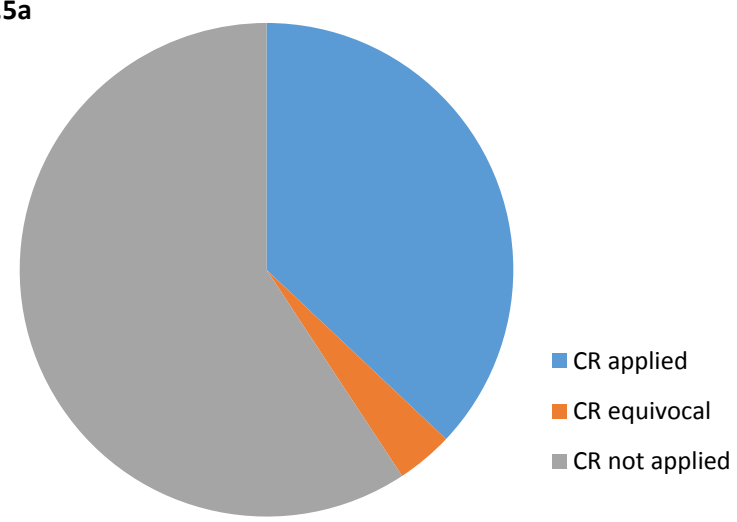
The remaining 9 biopsies, had been stained with Congo red locally but thought not to show amyloid. We were able to retrieve 5 of these biopsy samples; four were found to show

amyloid on Congo red staining at the NAC but 1 did not show green birefringence after Congo red staining although there was evidence of renal amyloid on the basis of EM, corroborated by SAP scintigraphy.

The diagnosis of MCD was made without EM in 17 of 26 patients in this series. In the remainder, EM was performed but was reported not to show fibrils thus supporting a diagnosis of MCD. Interestingly, 9/26 (35%) patients had neither Congo red staining nor EM performed prior to being diagnosed with MCD (Fig 3.5b). EM was retrospectively undertaken on 10 of 17 biopsy specimens that did not have EM performed initially. This revealed the presence of fibrils, typical of amyloid, in every case; all ten of these biopsy specimens were also found to contain amyloid by Congo red staining at NAC review.

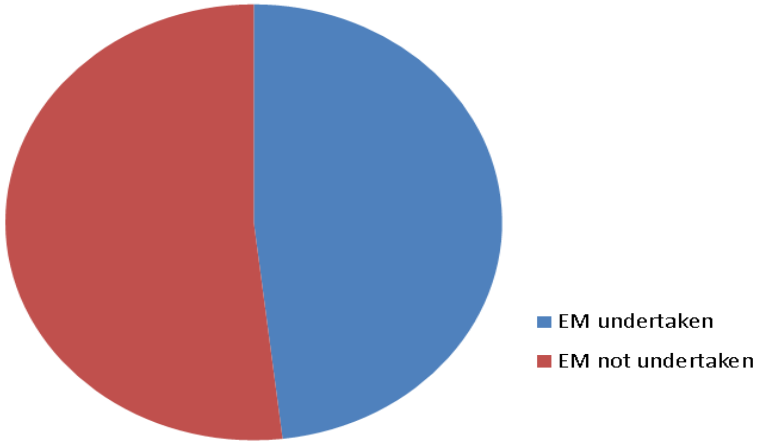
Fig. 3.5: Histological staining patterns. (a) Proportion of patients whose initial renal biopsy was stained with Congo red and those in whom the result was equivocal.

Fig. 3.5a



3.5b: Proportion of patients whose biopsies were reviewed under EM

Fig. 3. 5b



Sixteen patients underwent a second, and one a third renal biopsy before the diagnosis of amyloidosis was made. Fourteen of these 16 (88%) patients did in fact have demonstrable amyloid on their first renal biopsy when this was re-stained and/or reviewed at the NAC.

Discussion

I report here 27 patients with renal disease in whom an initial diagnosis of minimal change disease was revised to renal amyloidosis following reassessment of their kidney biopsies. This cohort highlights several distinct and important reasons for delays in establishing a diagnosis of renal amyloidosis. Seventeen of 26 available renal biopsies reported to have shown MCD had not been stained with Congo red, although each one was subsequently confirmed to contain amyloid deposits when sections were stained with Congo red and viewed under cross polarised light. Furthermore, EM images, available in 10/17 of these

biopsy specimens, showed the presence of characteristic fibrils. These data highlight the need for every renal biopsy specimen to be stained with Congo red and viewed under cross polarised light. A diagnosis of MCD cannot be made without the exclusion of amyloid using this 'gold standard' diagnostic test. EM should also be undertaken on renal biopsy specimens wherever possible, particularly for suspected MCD, with the presence of fibrils alerting the pathologist to the possibility of amyloid or alternative fibrillar pathology. It is noteworthy that in one of these 17 cases, Sirius Red staining was used. Although Sirius Red has been reported to result in 'stronger' staining that might be 'more noticeable' than Congo red, it is not associated with green birefringence under cross-polarised light and is thus inadequate for the exclusion of amyloid [280]. It remains uncertain whether these specimens were viewed under cross- polarised light or simply under brightfield light, but on review at the UK NAC, amyloid deposits were identified in each of 5 available tissue specimens; 4/5 on viewing the Congo red stained tissue under cross-polarised light, the remaining case showing characteristic fibrils on EM despite the absence of amyloid on Congo red. The diagnosis of renal amyloid in this last case was corroborated by abnormal renal uptake by SAP scintigraphy. These five cases highlight another likely important reason for failure to diagnose renal amyloid. Renal amyloid cannot be excluded by the absence of obvious extracellular eosinophilic material when viewed under brightfield light alone, even after Congo red staining of the specimen. It must be viewed in all cases under cross-polarised light.

There was a median time lapse of 241 days between the diagnoses of MCD and renal amyloidosis amongst these patients. The renal and overall outcomes in this group were poor with an eventual need for renal replacement therapy in 26% and median survival from diagnosis of amyloid of 4.7 years. AL amyloidosis is a progressive disease which in the absence of therapy is almost universally fatal, but chemotherapy directed towards the

underlying clonal plasma cell disease is increasingly successful. Prior to being diagnosed with renal amyloid, patients received steroids, often in combination with cyclophosphamide, calcineurin inhibitors or azathioprine in order to bring about resolution of their nephrotic range proteinuria. In each case, the reason for reviewing the initial kidney biopsy or undertaking a repeat kidney biopsy was a failure to respond to therapy. Taken together, these data support the need for the review of biopsy specimens in a Specialist Centre whenever the suspicion of renal amyloidosis remains high. Examples might include a patient with a PCD diagnosed with MCD who fails to respond to steroid therapy, or a patient with a PCD diagnosed with MCD who has significant cardiac or hepatic disease. Another situation in which review of histology by a Specialist centre is warranted, is when EM pictures show fibrils but the precise diagnosis remains unclear.

The case that failed to show the presence of amyloid, despite viewing of the Congo red stained tissue specimen under cross-polarised light, highlights some important issues. This patient presented with 9.1 g per 24 hours of glomerular proteinuria. EM confirmed the presence of randomly arranged, non-branching fibrils despite the absence of green birefringence on viewing of the Congo red stained tissue under cross- polarised light. One might postulate that the absence of amyloid on Congo red stain was due to 'sampling error' since this can be a patchy disease and the site of biopsy on this occasion may have been 'amyloid poor.' Alternatively, it may be that a certain number of fibrils are required before Congo red dye can intercalate sufficiently to demonstrate green birefringence under polarising filters and that this patient presented with proteinuria in the context of a 'pre-amyloid' state, as suggested by Hetzel [281]. Although amyloid fibrillogenesis by proteins must, by definition, involve a phase of pre-fibrillar aggregate formation, there is little evidence to support the idea of clinical disease before amyloid has supervened. It is thus more likely that sampling error is responsible for the failure to identify renal amyloid on the

very rare occasions that it may be missed despite correct staining of tissue specimens viewed under the correct conditions.

Treatment of amyloidosis with appropriate chemotherapeutic or biological agents in a timely fashion can halt ongoing amyloid deposition and prevent progression to ESRD and the requirement for renal replacement therapy [40, 282]. Furthermore, early diagnosis may mean a reduction in the likelihood of infectious and thrombotic complications associated with a prolonged nephrotic state [282]. Finally, correctly diagnosing renal amyloidosis will prevent inappropriate, ineffectual and potentially harmful use of drugs such as steroids, cyclophosphamide and/or CNIs. It is thus paramount that all kidney biopsy specimens are routinely stained with Congo red and viewed under cross-polarised light. In addition, renal tissue should be obtained for EM wherever possible, at the very least so that the pathologist can revert to analysis of EM pictures when the clinical picture demands it. Whenever the clinical suspicion of renal amyloidosis remains high, tissue blocks should be forwarded to Specialist Amyloidosis Centres.

Results and Discussion

Chapter 4: Mass Spectrometry in the Diagnosis of Renal Amyloidosis and its Subtypes

Aim

The pitfalls of current established diagnostic techniques, specifically immunohistochemistry (IHC), have been highlighted in the *'Introduction'* session. This study was undertaken to investigate whether a method could be established, namely proteomics, by which amyloidogenic variants, specifically Fibrinogen A α-chain, could be identified in a sensitive and specific manner.

Introduction

Amyloidosis is a disorder arising from the extracellular deposition of insoluble proteins, in a β-pleated form [13]. Examination under light microscopy reveals the presence of amorphous deposits that further stain red following the application of Congo red dye and, characteristically, demonstrate apple-green birefringence under cross-polarised light [265]. Electron microscopy further reveals the presence of rigid, non-branching fibrils 7.5 to 10nm in diameter and as explained more extensively in the introduction, this deposition eventually leads to both the structural and functional deformation of organs, with the exact pattern and severity of disease depending upon the subtype of amyloid fibril protein involved [22]; with AL amyloidosis and reactive or secondary AA amyloidosis being the commonest forms [283]. The current techniques employed to diagnose the amyloid subtype are not without significant pitfalls. IHC is not diagnostic in up to 50% and 10% of cases of AL and AFib amyloidosis respectively [94, 284]; this may be due to high background staining secondary to serum contamination and loss of epitope detectability in the tertiary structure, due to

protein cross-linking after formalin fixation [67] [253]. Mass spectrometry may prove to be a more sensitive and specific method by which to diagnose the particular amyloid subtypes [266] [267]. Furthermore, IHC requires the application of different antibodies on separate slides which is a labour-intensive process.

Renal amyloid deposition in AFib has a very typical appearance on light microscopy: typically there is extensive deposition of amyloid which is confined to the glomeruli and causes their enlargement and almost complete obliteration [151]. The tubulointerstitial space and vessels are, by comparison, almost completely spared. Immunohistochemical staining with antibodies is very specific for the fibrinogen Aα chain and a previous study of 71 patients by Gillmore et al., found the sensitivity to be 93%, with the remaining cases being identified through direct DNA sequencing, typical renal morphology and a family history and/or a disease course typical for AFib amyloidosis. In this group, SAP scintigraphy revealed tracer uptake in 89% of patients and splenic uptake in 21% [151]. Thus far, gene sequencing has led to the recognition of a range of fibrinogen A α-chain variants that give rise to this condition and there is evidence that the presenting age, severity of disease and prognosis varies between the different genotypes. There are currently 15 known amyloidogenic fibrinogen A α-chain variants arising from both single nucleotide substitutions and frameshift mutations: -Glu526Lys, Pro552His, Gly519Arg, Glu526Val, Gly555Phe, Thr538Lys, Arg554Leu, Arg554Leu, Glu540Val, Val522Alafs*27, Pro552His, Glu524Glufs*25 Phe521Serfs*27, Glu540Val). The first described and most common amyloidogenic variant is E526V, which arises from a single point mutation resulting in the substitution of valine for glutamic acid at position 526 of the mature protein [94].

As mentioned in the introduction, patients with AFib amyloidosis show a progressive worsening of function, typically presenting with nephrotic syndrome and hypertension in the

5th decade of life. CKD usually develops within 1-5 (median 4.6) years from the time of diagnosis and when progression to ESRD has occurred, there is an estimated survival time of approximately 9 years and survival from the time of presentation is typically 15 years [94]. Hence, the diagnosis of this rapidly progressive renal disorder is of the utmost importance in planning renal replacement therapy, including renal transplantation and considering the potentially curative procedure of hepato-renal transplantation [274]. It is also becoming increasingly evident that the identification of the particular variant is important in predicting disease trajectory, which may vary between different subtypes.

Methods Specific to this Study

Specimen Preparation and Microdissection

We undertook laser dissection and mass spectroscopy on 165 renal biopsies, received from centres that had referred patients to the NAC for diagnostic and treatment advice purposes. 10 μm thick sections of formalin-fixed paraffin embedded tissues were stained with Congo-Red dye. Samples that stained with the dye were first identified under bright field light and then viewed under cross-polarised light to demonstrate the previously discussed tinctorial properties that are unique to amyloid fibrils. Immunohistochemical stains were performed with the aid of a DAKO Autostainer (Dako North America Inc., Carpinteria, CA, USA) using the Dual Link Envision+ or ADVANCE (Dako) detection systems. Antibodies were directed against the following antigens, with the corresponding clones for the monoclonal antibodies specified: CD3 (Novocastra, Newcastle, UK; clone PS1; dilution 1:50), CD20 (Dako; clone L26; dilution 1:60), CD68 (Dako, clone KP-1; dilution 1:3000), CD138 (Dako; clone M115; dilution 1:50), Kappa Free Light Chains (Dako, polyclonal, dilution 1:6000), Lambda Free Light Chains (Dako; polyclonal; dilution 1:2000), Lambda Light Chain (Auto ProEnzyme pretreatment; Dako; polyclonal; dilution 1:3000), Prealbumin (Transthyretin) (Dako; polyclonal; dilution 1:5000), Serum Amyloid A (SAA; Dako; clone MC-1; dilution 1:1000),

and Serum Amyloid P component (SAP; Biocare; polyclonal, dilution 1:20) and Fibrinogen (FB) 1:400 camb bioscience.

Laser section microdissection and protein identification via mass spectrometry was undertaken as described previously in the *Material and Methods'* section.

PCR and Direct Sequencing of the *FGA* gene

Genomic DNA was extracted from whole blood treated with EDTA as previously described [273]. A 707-base pair fragment of exon 5 of the fibrinogen α-chain gene (*FGA*) was amplified by polymerase-chain-reaction assay (PCR) and analysed by automated sequencing. PCR was carried out with Ready-To-Go tubes (Amersham Pharmacia Biotech) with the use of solutions and cycling conditions that have been previously described [285]. The PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol and sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the procedures recommended by the manufacturer. Sequence of the *FGA* gene was analysed on the ABI 3130xl Genetic Analyser using Sequencing Analysis Software version 5.4.

Results

From these 165 biopsies, 58 patients were identified by microscopy, to have isolated glomerular amyloid. Light microscopy, staining with antibodies and gene sequencing revealed that 32 of these patients had AFib and the remaining 26 'controls' had other amyloid subtypes. Mass spectrometry was undertaken on all biopsies and revealed that the 'amyloid signature' was present in all cases (Table 4.1) i.e. ≥2 of apolipoprotein E, serum amyloid P component and apolipoprotein A-IV.

Table 4.1 Amyloid signature proteins

	N	ApoE	SAP	ApoA-IV
AFib	32	32 (100%)	32 (100%)	14 (44%)
Control	26	26 (100%)	25 (96%)	14 (54%)

Results revealed multiple potential amyloid fibril proteins in most samples (table 4.2). Hence, it was important to identify the actual variant peptide of fibrinogen A α-chain in order to confirm the diagnosis of AFib amyloidosis.

Table 4.2 Amyloid fibril proteins identified

	N	FibAα variant present	N
AFib	32	32 (100%) E526V(pGlu545Val) E524K(pGlu543Lys) P552H(pPro571His) R554L(pArg554Leu)	29 1 1 1
Controls	26	0 (0%)	
	N	**FibAα**	**Other amyloid fibril proteins**
AFib	32	32 (100%)	23 (72%)
Controls	26	11 (42%)	26 (100%)

An example of how variant peptides were identified when the sequences for known fibrinogen variants were added to the Uniprot database, is given below (Fig. 4.1):

4.1a.

```
  1 MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS
 51 DWPFCSDEDW NYKCPSGCRM KGLIDEVNQD FTNRINKLKN SLFEYQKNNK
101 DSHSLTTNIM EILRGDFSSA NNRDNTYNRV SEDLRSRIEV LKRKVIEKVQ
151 HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC RGSCSRALAR EVDLKDYEDQ
201 QKQLEQVIAK DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ LQKVPPEWKA
251 LTDMPQMRME LERPGGNEIT RGGSTSYGTG SETESPRNPS SAGSWNSGSS
301 GPGSTGNRNP GSSGTGGTAT WKPGSSGPGS TGSWNSGSSG TGSTGNQNPG
351 SPRPGSTGTW NPGSSERGSA GHWTSESSVS GSTGQWHSES GSFRPDSPGS
401 GNARPNNPDW GTFEEVSGNV SPGTRREYHT EKLVTSKGDK ELRTGKEKVT
451 SGSTTTTRRS CSKTVTKTVI GPDGHKEVTK EVVTSEDGSD CPEAMDLGTL
501 SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF VSETESRGSE
551 SGIFTNTKES SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS
601 YKMADEAGSE ADHEGTHSTK RGHAKSRPVR DCDDVLQTHP SGTQSGIFNI
651 KLPGSSKIFS VYCDQETSLG GWLLIQQRMD GSLNFNRTWQ DYKRGFGSLN
701 DEGEGEFWLG NDYLHLLTQR GSVLRVELED WAGNEAYAEY HFRVGSEAEG
751 YALQVSSYEG TAGDALIEGS VEEGAEYTSH NNMQFSTFDR DADQWEENCA
801 EVYGGGWWYN NCQAANLNGI YYPGGSYDPR NNSPYEIENG VVWVSFRGAD
851 YSLRAVRMKI RPLVTQ
```

4.1b

```
  1 MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS
 51 DWPFCSDEDW NYKCPSGCRM KGLIDEVNQD FTNRINKLKN SLFEYQKNNK
101 DSHSLTTNIM EILRGDFSSA NNRDNTYNRV SEDLRSRIEV LKRKVIEKVQ
151 HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC RGSCSRALAR EVDLKDYEDQ
201 QKQLEQVIAK DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ LQKVPPEWKA
251 LTDMPQMRME LERPGGNEIT RGGSTSYGTG SETESPRNPS SAGSWNSGSS
301 GPGSTGNRNP GSSGTGGTAT WKPGSSGPGS TGSWNSGSSG TGSTGNQNPG
351 SPRPGSTGTW NPGSSERGSA GHWTSESSVS GSTGQWHSES GSFRPDSPGS
401 GNARPNNPDW GTFEEVSGNV SPGTRREYHT EKLVTSKGDK ELRTGKEKVT
451 SGSTTTTRRS CSKTVTKTVI GPDGHKEVTK EVVTSEDGSD CPEAMDLGTL
501 SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF VSETVSRGSE
551 SGIFTNTKES SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS
601 YKMADEAGSE ADHEGTHSTK RGHAKSRPVR DCDDVLQTHP SGTQSGIFNI
651 KLPGSSKIFS VYCDQETSLG GWLLIQQRMD GSLNFNRTWQ DYKRGFGSLN
701 DEGEGEFWLG NDYLHLLTQR GSVLRVELED WAGNEAYAEY HFRVGSEAEG
751 YALQVSSYEG TAGDALIEGS VEEGAEYTSH NNMQFSTFDR DADQWEENCA
801 EVYGGGWWYN NCQAANLNGI YYPGGSYDPR NNSPYEIENG VVWVSFRGAD
851 YSLRAVRMKI RPLVTQ
```

Fig 4.1. The most common mutation (p.Glu545Val or position 545 of the mature protein), This involves a single base substitution that alters the codon at position 526 of the mature protein from that for glutamic acid (GAG) to valine (GTG), as demonstrated by the circled *area (Fig 4.1a). Primary search with results using the standard Uniprot database, 4.1b. Secondary search with results using the Uniprot database with the variant database appended, reveal the single mutation at position 545.*

Results following findings post-searches using the Uniprot database with the additional database of known fibrinogen variants appended, are summarised in Table 4.3.

Table 4.3 Gene sequencing was used to compare the presence or absence of a variant in each case.

	N	FibAα variant present	N
AFib	32	32 (100%)	
		E526V(pGlu545Val)	29
		E524K(pGlu543Lys)	1
		P552H(pPro571His)	1
		R554L(pArg554Leu)	1
Controls	26	0 (0%)	

Discussion

This study demonstrates that proteomics can be used to successfully identify AFib amyloidosis on the basis of the presence of the variant peptide. The current techniques employed i.e. IHC and then gene sequencing are not without their own difficulties. Problems encountered include non-binding of antibodies to epitopes that may be 'hidden' such that the amyloid subtype cannot be identified [286]. Also a constellation of antibody tests is undertaken on each sample and this process can be avoided if mass spectrometry is used. The step of gene sequencing can also be omitted once a sufficiently large database of fibrinogen variants is acquired. However proteomics, at present, has its own limitations and although used as an adjunct in particularly difficult cases, it is not currently being used in isolation for identification of amyloid subtypes and particular genetic variants. Problems encountered include: the easy introduction of background contaminants for example, if one did not have a fibrinogen database for variants, one would see fibrinogen in any number of

samples that have been contaminated with blood, not an uncommon occurrence. Wild-type fibrinogen A α-chain is not amyloidogenic so that identification of a variant peptide confirms AFib amyloidosis, as compared to ATTR in which the wild-type protein does form amyloid. The formalin fixation process can chemically alter proteins so reducing the efficacy of protease cleavage and/or altering the mass of resulting peptides. The timely processing of a large number of samples is also hindered by the need for a rate-limiting overnight trypsin digestion step. The variables discussed must be considered in the bioinformatics analysis to ensure accurate protein identification. Moreover, current quantitative analysis methods are based on the spectrum count, which ignores differences in protein length and may lead to misdiagnosis i.e. larger proteins generate more peptides.

RESULTS AND DISCUSSION

Chapter 5: The Usefulness of Urinary Retinol Binding Protein Measurements in Amyloidosis

Aim

To investigate whether urinary retinol binding protein could potentially serve as a novel sensitive and specific biomarker for renal injury across the amyloid subtypes and, furthermore, provide prognostic information.

Introduction

AL amyloid, as previously mentioned, is the most prevalent of the amyloid subtypes and the kidney can be affected in 50-80% of cases. Not all subtypes affect the kidneys, whereas some subtypes exclusively deposit in the kidneys alone, with notable hereditary forms including Fibrinogen Aα, Gelsolin, ApoAI, ApoAII and 'acquired' types including AL amyloidosis arising from amyloidogenic light chains and AA amyloidosis, the latter arising from a chronic inflammatory source [95, 287, 288]. Proximal tubular involvement varies upon the subtype involved with renal impairment progressing less rapidly when tubulointerstitial rather than glomerular deposition predominates with heavier proteinuria being associated with glomerular lesions [31, 289]. Another extraglomerular manifestation of renal amyloidosis is Fanconi's syndrome, reflecting injury to the proximal tubular cells (PTCs) by filtered light chains in the case of AL amyloidosis [290, 291]. There are 2 possible mechanisms by which light chains are believed to promote tubular toxicity. One is through their inherent toxicity towards PTCs, including tubulointerstitial inflammation and fibrosis, noted *in vitro* and *in vivo* and the other is through crystallisation in the proximal tubular

cytoplasm [292-296]. It is thought that injury to the PTCs by filtered LCs reduces the capacity of the tubules to reabsorb glucose, uric acid, bicarbonate and amino acids from the glomerular filtrate. This alteration in reabsorptive function produces Fanconi's syndrome, the most readily identifiable signs of which are glucosuria and metabolic acidosis [291, 292]. More recently, work by Luciani et al, postulates that light chains accumulate in PTC lysosomes and alter their function leading to defective acidification so that the apical transport capacity of PTCs is lost [294].

A 'biomarker', derived from the term 'biological marker,' has been defined by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [297]." The attributes of an ideal biomarker have previously been summarised and are widely accepted as including: being non-invasive, identifying the risk of developing an illness (antecedent role) including identifying subclinical disease (subclinical role), being sensitive and specific in diagnosing disease (diagnostic role), being robust, correlating rapidly and reliably with the degree of organ/process injury (staging and risk-stratification role), providing prognostic information (prognostic role), being site specific, being applicable across different populations, identifying possible mechanisms of injury e.g. prerenal, intrarenal, postrenal, being highly stable over time, different temperatures and pH and not interfering with drugs and being reproducible [298-301].

There are a number of new biomarkers being identified and investigated, potentially to replace or aid the diagnostic and prognostic information obtained through an invasive biopsy. Currently, the only widely established and accepted renal biomarkers, not just in amyloidosis but in general nephrology, are serum creatinine (including the derived eGFR)

and urinary protein loss. Specifically, in AL amyloidosis, proteinuria >5 g/24h and eGFR <50 mL/min predicted progression to dialysis best, as previously described [116]. Urinary RBP is being investigated as an important and sensitive biomarker for the loss of function of the human proximal renal tubule. It may also have a possible association with the extent of interstitial kidney fibrosis [296]. Damage to the proximal tubule leads to 'tubular' proteinuria as smaller proteins including RBP (21 kDa) are readily filtered by the glomerulus and appear in the urine when they fail to be re-absorbed in the proximal tubule. In the 'healthy' state the fractional tubular reabsorption of RBP is 99.97% and the presence of urinary RBP is therefore a sensitive marker of tubular dysfunction [296]. Marked tubular proteinuria with very high urinary RBP levels is found in hereditary forms of Fanconi's syndrome, which include Dent's disease, Cystinosis and Lowe syndrome. Lower levels are sensitive indicators of the carrier status of these hereditary disorders and also of toxic tubular damage [255, 295, 296]. Most recently, Li et al., retrospectively studied 119 AL amyloidosis patients and found that RBP had a significant positive correlation with blood urea nitrogen, serum creatinine, urinary κ light chains and urine protein and a significant negative correlation with creatinine clearance, eGFR and serum albumin [255]. However, they did not look at other forms of amyloidosis and it would be interesting to note whether there is any correlation with forms of amyloidosis other than AL.

We sought to determine whether renal amyloidosis was associated with tubular proteinuria and in particular, whether non-AL renal amyloidosis was associated with evidence of proximal tubular dysfunction, in order to determine the relative contribution to tubular dysfunction from amyloid deposits as opposed to light chains. Further study will also help to determine whether urinary RBP has a role in predicting renal outcome among patients with a range of amyloid types.

Material and Methods Specific to this Study

81 patients with known or suspected renal amyloid had fasting samples taken for renal profile, bone profile, serum glucose, glucosuria, urinary electrolytes and creatinine, 24 hour urine collection for proteinuria and spot urine RBP measurements performed between 6th November 2014 and 19th December 2014. In this study, the urinary RBP/creatinine ratio (RCR) was used as opposed to just the just the urinary RBP to account for differences in concentration of the urine. Patients were included in the study as they appeared on a routine clinic list between those dates i.e. there was no pre-selection. All patients were managed in accordance with the Declaration of Helsinki, and informed patient consent and institutional review board approval from the Royal Free Hospital Ethics committee were obtained for this study.

Analysis of Samples

Urine samples immediately underwent urinalysis and aliquots were then stored at -80 degrees Celsius without preservative and within an hour of collection, to reduce the risk of microbial contamination. They were analysed for urinary RBP and creatinine within a week of the study sample collection being completed and samples were thawed to room temperature only at the time of analysis. The analysis of urinary RBP was undertaken using a very sensitive immuno-nephelometric technique involving the use of latex enhanced antibodies (the Binding Site Group Limited, Birmingham), according to the manufacturer's protocol and employing an ELISA technique [268]. The reference range for urinary RBP/creatinine ratio (RCR) using this method is 3.9-32 ug/mmol for children aged 10-16 years and there is no adult reference range for this RBP method, although previous studies have found the upper limit of normal to be 17ug/mmol [302]. The Siemens BNII Nephelometer was used. Urine samples were centrifuged prior to assaying and controls were run with every batch of samples. All samples and calibrators were analysed in duplicate

and the mean absorbance results were plotted against calibrator concentration. Any samples with an absorbance greater than the top calibrator were re-assayed using a greater dilution. The measured values were normalised to the urinary creatinine concentration. The within batch coefficient of variation was <2% and the between batch coefficient of variation was < 5%, across the assay range. Urinary creatinine was measured using the Jaffé method.

Stick urinalysis was performed using Multistix (Bayer Diagnostics), according to the manufacturer's instructions and using an automated reader by the same person throughout the study. The degree of glucosuria was classified as negative, trace or more than trace (>100mg/dL).

The glomerular filtration rate was estimated using the Cockcroft-Gault equation (eGFR) [303]. Renal improvement or deterioration was defined as a 25% decrease or increase in serum creatinine or an increase or decrease in modified eGFR respectively, as per Gertz et al. [106]. The mean eGFR at initiation of renal replacement therapy (RRT) in 2013 was 8.5 ml/min/1.73 m^2 [304] and this was taken as the figure where patients started RRT. Patients who did not fulfil the criteria for renal progression or deterioration were deemed to have stable kidney function.

SAA levels were assayed with the use of latex-enhanced immunonephelometry (BN II analyser, Dade Behring) using the World Health Organisation's International Reference Standard.

The renal tubular maximum reabsorption rate of phosphate to the glomerular filtration rate (TmP/GFR) was calculated [305], with values being derived from a fasting serum phosphate

and creatinine and a spot urinary phosphate. The normal range was taken as per the study by Minospla et al [306], with male or females >55 years old having a TmP/GFR of 0.80-1.35.

Amyloid Load

Quantitation of visceral amyloid deposition was by whole-body ^{123}I-labeled SAP scintigraphy, as previously described [144]. Resulting images were assessed by two independent specialists and graded as a small, moderate or large load.

Renal Biopsy Specimens

Specimens for light microscopy were fixed in 10% formaldehyde (formalin) and embedded in paraffin, sectioned, and stained with haematoxylin-eosin, periodic acid–Schiff, Masson trichrome, and Jones silver methenamine. Specimens were initially assessed at the referring hospital and then sent to the National Amyloidosis Centre where they were further reviewed, alongside unprepared tissue samples. Tubular atrophy (TA) was graded in accordance to the percentage of atrophy seen in the total biopsy by 2 independent observers as per the MEST criteria in the Oxford Classification, with 0-25% being mild (T0), 26-50% (T1) being moderate and above 50% (T2) being severe [307]. In 7 samples, the amount of renal material was insufficient to accurately assess this and 2 biopsies were of extra-renal tissue i.e. liver and fat.

All unprepared tissue samples were fixed in formalin and embedded in paraffin. Serial sections were stained with haematoxylin and eosin and six µm-thick renal sections were cut and stained with alkalinised Congo Red by the method developed by Puchtler et al. [265]. All such samples along with previously stained slides were viewed under cross-polarised light by two independent observers at the NAC to determine the presence of renal amyloid deposits. Immunohistochemistry was performed with commercially available monoclonal

antibodies directed against AA amyloid, polyclonal antibodies against amyloid P-component, fibrinogen, lysozyme, transthyretin, λ-light chain, κ-light chain as well as non-commercially available polyclonal antibodies directed against Apo A1, as described previously [284].

DNA Sequence Analysis

Genomic DNA was extracted from whole blood treated with EDTA as previously described and the following genes were sequenced; LECT2, Fibrinogen Aα chain, APOA1, APOA2, lysozyme [308].

Statistics

Data were analysed using IBM SPSS Statistics version 19 (IBM, Somers, NY) and data are presented either as mean +/- SD or SE or median (range) for continuous variables. Ordinal variables are presented as frequency and percentage. Pearson two-variable correlation analysis was used between RCR and eGFR, proteinuria, TmP/GFR and serum albumin. Spearman's rho *"r"* rank correlation co-efficient was used to analyse the correlation between RCR and amyloid load, glucosuria, TA and LC levels. A p value of <0.05 was taken as being statistically significant. Mean survival and SE was calculated by using Kaplan-Meier and follow-up and SE was calculated using reverse Kaplan-Meier. The Mann-Whitney U test was used in finding a difference in RCR values between different pathognomonic LCs in AL amyloidosis.

Results

81 patients were included in the study, 79 of those patients had a renal amyloid subtype, 1 had light chain deposition disease (LCDD) and the other had MCD (table 5.1). The average age at inclusion of study was 65.9 years and the median follow up period by reverse Kaplan-

Meier was 1.65 years (standard error 0.014). Mean survival by Kaplan-Meier was 1.61 years and standard error 0.04, with 7 patients having died during follow up. 51 (62.96%) of the patients were male.

Further analyses were performed on the 79 patients with amyloidosis. Pearson two-variable correlation analysis between RCR and other laboratory characteristics showed that RCR had a significant and moderate negative correlation with eGFR only ($r=-0.43$ and $p<0.001$) and no significant correlation with serum albumin and proteinuria. In fact, there was no significant correlation between proteinuria and RCR in any of the various amyloid subtypes (RBP correlation for proteinuria. AA, $r=0.506$ and $p=0.136$; AL, $r=0.144$, $p=0.272$; Fib Aα, $r=0.748$. $p=0.087$; LECT2, $r=0.027$, $p=0.983$). Spearman's rank correlation co-efficient was used to analyse the correlation between RCR and amyloid load (as seen on the SAP scan), glucosuria, TA and a change in eGFR at the end of the study. There was a significant negative correlation seen between RCR and glucosuria and a significant and moderate positive correlation between RCR and the degree of TA. There was no correlation between RCR and change in eGFR ($p=-0.033$ and $p=0.785$).

Table 5.1 Laboratory and imaging characteristics at baseline

Diagnosis	No. of Pts	Median eGFR (ml/min)	Median proteinuria (g/24hr)	Median RCR (range)	Amyloid Load* N/S/M/H (%)	TmP/GFR Below NR (%)	Glycosuria† N/T/>T (%)
AL	60	39	2.78	66.65 (7.1-38675)	23/47/22/8	22	87/5/8
AA	10	26	3.31	2689 (8-9577)	0/40/60/0	20	60/30/10
Fib Aα	6	23.5	23.5	696.9 (44.8-10540)	0/100/0/0	0	67/0/33
LECT2	3	53.33	0	35 (3.6-1018)	0/67/33/0	33	3/0/0
LCDD	1	44	0.83	74.7	N/A	0	100/0/0
MCD	1	>90	1.2	206	N/A	0	100/0/0

*Amyloid Load: N= None, S=Small, M=Moderate and H=Heavy

†Glucosuria: N=None detected, T=Trace detected, >T=greater than trace detected

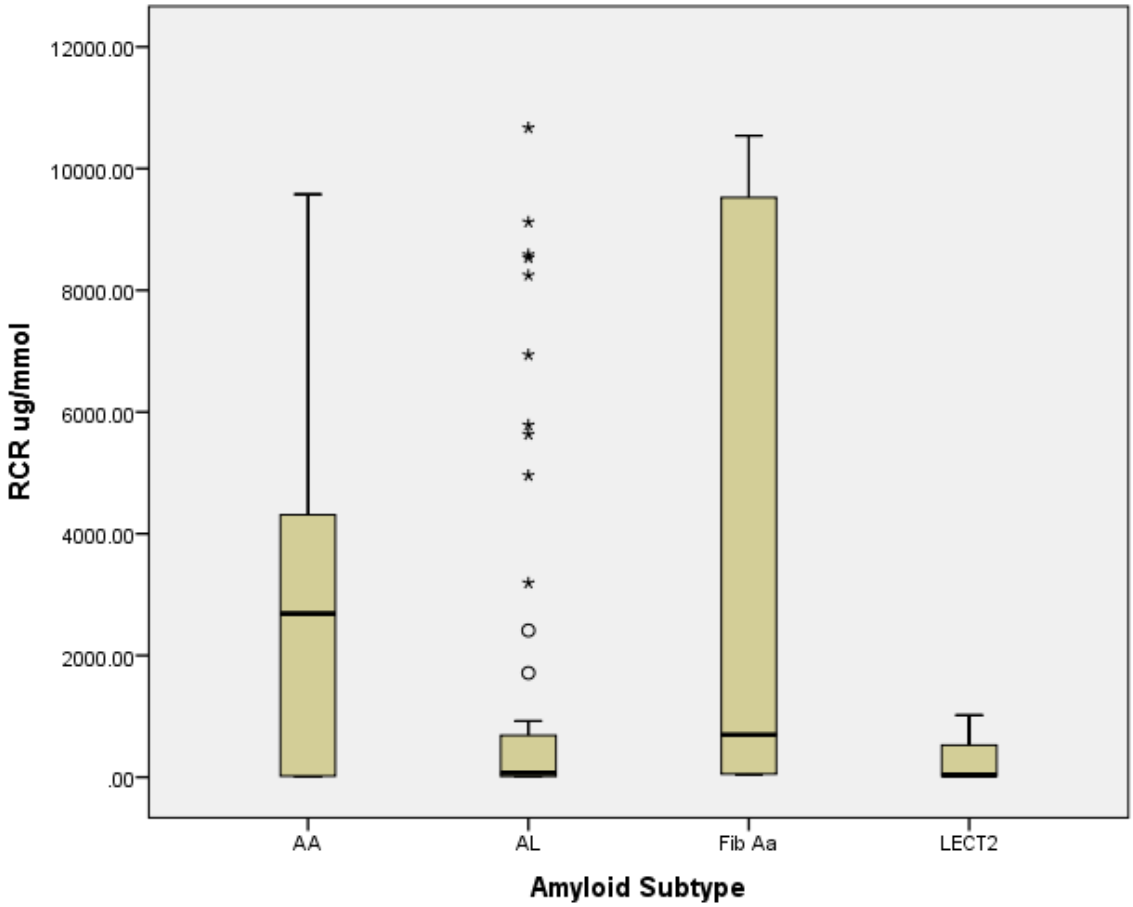
Amyloid load: None, Small, Moderate, Heavy (N/S/M/H); LCDD: Light Chain Deposition Disease; MCD: Minimal Change Disease; NR Normal Range

Table 5.2 Correlation between RCR, serum eGFR, proteinuria, amyloid load, glucosuria, serum albumin, TmPGFR and tubular atrophy at baseline- for all patients with amyloid

	eGFR	Proteinuria	Load on SAP	Glucosuria	Serum Albumin	TmPGFR	Tubular Atrophy
Correlation Co-efficient	-0.43	-0.19	0.06	0.65	-0.05	-0.301	0.450
Significance (2- tailed)	0.000064	0.093	0.6	5.93x10-11	0.659	0.007	<0.01

The parallel box plots below demonstrate the comparative lie of data across the different amyloid subtypes, asterisked data denote outliers 1.5x greater than that demonstrated by the 'range' of data.

Fig 5. A comparative box plot showing median and interquartile ranges across the amyloid subtypes with circled and asterisked data denoting outliers.



In order to better appreciate the lie of distribution, an AL outlier with an RCR value of 38 675 ug/mmol has not been shown.

Analysing just the AL group, the mean RCR level for patients with an amyloidogenic Kappa (n=10) LC pathology was 4921.1 (s.d. =12148.96) and for amyloidogenic Lambda (N=50) LC pathology was 1425.4, however this was not a significant difference (U=190.000, p=0.23). However, RCR values did significantly correlate positively with Lambda LC levels (r=0.434, p= 0.002) and had borderline significance for Kappa LCs (r=0.624, p=0.054), the

p value being influenced by the relatively small number of patients with pathogenic light chains (n=10 for Kappa).

Discussion

The values of RCR obtained in the study ranged from 3.6 to 38 675 µg/mmol and to date there are no studies detailing RCR results in the amyloid population, with the most recent study by Li et al, using RBP values alone and only for patients with AL amyloid. A study in the paediatric population reveals that RCR varied between 1.99 to 376 µg/mmol for MCD (not dissimilar to the single MCD patient in this study, who had an RCR of 206) and focal segmental glomerulosclerosis (FSGS) respectively. The range in the FSGS population was from 6.9 to 20398 µg/mmol [308]. The highest RCR value in this study, 38 675 µg/mmol, was for a patient with AL amyloid, an eGFR of 18, proteinuria of 0.27g/24h, significant levels of glucosuria and TmP/GFR of 0.63, a Lambda light chain level of 106 and perhaps unsurprisingly, severe TA on renal biopsy. These features reflect the general findings of this study, that is:

A higher RCR value was correlated with:

- worsening eGFR
- a greater degree of glucosuria
- a lower TmP/GFR i.e. decreased tubular phosphate reabsorption
- more severe TA
- higher levels of LCs (p=0.002 for Lambda LC levels and p=0.054 for Kappa LC levels)

We found no correlation between RCR and:

- Change in eGFR over the course of the study
- Load on SAP scintigraphy at baseline
- Proteinuria
- The type of LC (for patients with AL amyloidosis)
- And serum albumin

Higher RCR values are thought to reflect worsening tubular dysfunction, as demonstrated by *in vitro* and *in vivo* studies and a feature of some tubular disorders is Fanconi's syndrome- characteristics of which can include a low TmP/GFR and glucosuria- as demonstrated by the findings of this study. Worsening tubular function would also tend to reflect disease activity in the kidneys as a whole and therefore the negative correlation with eGFR is again unsurprising. Those with RCR values in normal range, taken as <32 ug/mmol (n=29) and those with a RCR ≥ 32 µg/mmol (n=50) had a significant difference in mean eGFR (p=<0.001) with a mean eGFR of 73.86 and 36.84 mL/min/1.73 m^2 respectively.

As seen on the parallel box plots, the median values for AA amyloid were greater than the other amyloid subtypes although the interquartile range was similar to FibA amyloid. This is perhaps surprising considering that pathogenic light chains, as in AL amyloid, are believe to be toxic to the renal tubules but other amyloid fibril proteins are not known to be, suggesting that the amyloid itself is likely to disrupt tubular function. However, we could find no previous studies of non-AL amyloidosis for comparison. The median SAA level was 16.5 mg/L (range <3.2-41.9) and it is known that amyloid regression occurs in approximately 60% of patients when SAA levels are <10mg/L [40]. There was no correlation in this cohort between SAA levels and RCR values (r=-0.004, p=0.991). Differences in the degree of TA identified on renal biopsy between AL and AA patients also does not account for the higher RCR values found (p=0.513 not assuming equal variances), realising of course the sampling error

present in any biopsy. It may be that an ongoing inflammatory process *per se*, has a disruptive effect on tubular transport mechanisms as we know that tubular epithelial cells are targeted by inflammatory mediators which can lead to cell necrosis, as discussed in the review by Cantaluppi et al. [309]. The inflammatory mediators can also work indirectly to damage tubular function by reducing renal perfusion and lead to ischaemia-reperfusion injury. Lachmann et al. in their observational study on AA amyloid discuss that the molecular mechanisms of tissue damage in AA amyloid remain unknown and are affected by additional insults including drug, sepsis, hypovolaemia and hypertension- so that actually these may account for tubular injury rather than the presence of raised SAA levels [40, 310].

The analysis of these results for patients with hereditary amyloid is limited by their relatively small number. However, our findings indicate that further study of a larger sample of this population is warranted. In terms of the prognostic value of RCR, there was no correlation between eGFR change and baseline RCR values, although one could extend the study further. Additionally, this was a prospective observational study and uncontrolled for treatment regimens i.e. there was variation in treatment received (if any) between patients with the same disease, such as chemotherapy regimes/ stem cell transplantation in AL amyloid and anti-inflammatories in AA amyloid. Controlling for these variables which are known to influence renal prognosis and outcome require large studies since the degree of suppression of the relevant fibril precursor protein (i.e. response to treatment) varies substantially between individual patients and is also dependent on other factors e.g. the extent of cardiac amyloidosis. It may very well be that the treatments themselves were tubulo-toxic and therefore would affect any prognostic association that baseline RCR values may have with changes in eGFR.

There was no correlation between amyloid load as seen on SAP scintigraphy and RCR which is perhaps unsurprising as radioactive tracer uptake and subsequent renal tissue

distribution will be affected by declining eGFR. Hence, the burden of renal amyloid disease cannot simply be delineated from SAP scintigraphy.

The correlation between higher LC levels and RCR values in patients with AL amyloidosis supports previous studies demonstrating the direct tubular toxicity caused by LCs.

This study demonstrated that RCR values at baseline can add to the information provided by current biomarkers but cannot replace their usage. It also suggests that RCR values at baseline cannot be used to prognosticate changes in eGFR although further study including larger patient numbers is required to confirm this due to the large number of confounding variables such as the presence of cardiac amyloid, infections, effect of chemotherapy or other treatments. It also demonstrates that amyloid patients as a whole tend to have high RBP values and that higher RBP values correlated significantly with lower eGFR values. Surprisingly, patients with Fib A and AA amyloid tended to have higher RBP values. We would advise further studies with a greater number of patients with hereditary amyloid and AA amyloid, to see if our results can be reproduced.

RESULTS AND DISCUSSION

Chapter 6: Implanted Cardiac Rhythm Recorders in Advanced Cardiac AL Amyloidosis

The publication arising from this chapter is:

A study of implanted cardiac rhythm recorders in advanced cardiac AL amyloidosis.

Sayed RH, Rogers D, Khan F, Wechalekar AD, Lachmann HJ, Fontana M, Mahmood S, Sachchithanantham S, Patel K, Hawkins PN, Whelan CJ, Gillmore JD.

Eur Heart J. 2015 May 7;36(18):1098-105

My contribution was designing, undertaking, analysing results and writing up the study.

Aim

I sought to characterise the nature of, and disease-related risk factors for, cardiac arrhythmias in patients with severe cardiac AL amyloidosis by offering insertion of an implantable loop recorder (ILR) to a series of consecutive patients with newly diagnosed Mayo stage III cardiac AL amyloidosis attending the UK National Amyloidosis Centre. I hoped that such a study would reveal possible new therapeutic strategies in this cohort of patients, who are known to have the worst prognosis in AL amyloidosis.

Introduction

Deposition of AL amyloid may occur in almost any organ but involvement of the heart, present in more than 50% of patients at diagnosis, is associated with a poor prognosis [311]. The presence and severity of cardiac AL amyloidosis is assessed through serum biomarker

concentration, echocardiography and CMR [28, 131]. Approximately one third of patients diagnosed with systemic AL amyloidosis present with cardiac failure and marked elevation of the cardiac biomarkers NT-proBNP and TnT; despite major recent advances in chemotherapeutic strategies for treating AL amyloidosis, these patients frequently die of SCD within 12 months of diagnosis [123]. Although ventricular tachyarrhythmias and PEA have been observed as terminal events, little is known about the electrical activity preceding terminal cardiac decompensation (129, 184, 185). There has been considerable interest for over 30 years in whether certain interventions such as anti- arrhythmic or chemotherapeutic drug therapy, or the implantation of PPMs or ICDs might improve survival in cardiac AL amyloidosis [186]. Unfortunately, due to the exceptionally poor prognosis of those with advanced (Mayo stage III) cardiac AL amyloidosis, they are rarely included in prospective studies of novel chemotherapeutic agents [182, 312].

Materials and Methods Specific to this Study

Patients

Between July 2013 and May 2014, all patients attending the UK National Amyloidosis Centre with untreated, biopsy proven systemic AL amyloidosis who had an echocardiogram which was characteristic of cardiac infiltration by amyloid in conjunction with biomarkers indicating Mayo Stage III disease, and symptoms of either pre-syncope or syncope, were offered insertion of an ILR in accordance with National Institute of Health and Clinical Excellence (NICE) guidance. None of the patients had previously received a cardiac device.

Baseline Clinical Assessments

A comprehensive clinical consultation was undertaken in each case. Further evaluation including an ECG, transthoracic echocardiogram including tissue Doppler imaging, whole

body ^{123}I-serum amyloid P component scintigraphy scan, standardised 6 min walk test, lying and standing blood pressure measurements, NYHA and ECOG grading. Serological investigations included identification and quantitation of any circulating paraprotein and measurement of serum free light chains. First degree heart block and interventricular conduction delay were defined according to standard criteria (304, 305). Diastolic dysfunction on echocardiography was graded as mild, moderate, or severe and left ventricular (LV) strain was determined by tissue Doppler imaging, as previously described (306, 307).

Patient Treatment and Follow up

All patients received chemotherapy as standard treatment for systemic AL amyloidosis. All medications including details of each chemotherapy regimen and any changes in dosage were recorded at baseline and throughout the follow up period.

Where relevant, time to death was recorded and every effort was made to obtain clinical details and ILR recordings of the terminal event. Where these showed a rhythm compatible with a cardiac output in the absence of such an output, the cause of death was attributed to PEA. The censor date for surviving patients was 1st October 2014.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 statistical software package. Cox regression was used to determine factors at baseline associated with death. Hazard ratios and 95% confidence intervals were expressed per unit rise in each variable. Estimated median follow-up time was calculated by the reverse Kaplan–Meier method, and median patient survival was calculated by Kaplan–Meier analysis.

Results

Patients

Baseline characteristics of 20 consecutive patients with untreated, histologically proven systemic AL amyloidosis and Mayo stage III cardiac biomarkers are shown in Table 6.1. Median serum high sensitivity TnT was 0.13 µg/L and median NT-proBNP concentration was 1410 pmol/L; 13 patients had NT-proBNP >1003 pmol/L (>8500 pg/mL), known to be associated with a particularly poor prognosis [123]. Organ involvement by amyloid, assessed by amyloid consensus criteria and by SAP scan within one day of ILR placement, is shown in Table 6.1 [106]. At baseline, six patients were receiving beta-blocker therapy, one was receiving amiodarone and another took flecainide whenever he had symptomatic paroxysms of atrial fibrillation. Ten patients were receiving renin-angiotensin system (RAS) blockade and 16 were receiving loop diuretics (median daily furosemide dose equivalent 40 mg) of whom two were additionally receiving 50 mg daily of spironolactone.

Table 6.1 Baseline characteristics of patients at the time of ILR placement, including comparison between surviving and deceased patients

Characteristic		All patients (*N* = 20)	Deceased (*N* = 13)	Alive (*N* = 7)	Hazard ratio of death (95% CI); *P*-value
Female	*N* (%)	5 (25)	3 (23)	5 (71)	1.254 (0.340–4.619); 0.734
Age (years)	Median (range)	60 (34–78)	55 (34–75)	60 (55–78)	0.990 (0.937–1.045); 0.710
Mean arterial pressure (mmHg)	Mean (range)	92 (70–110)	92 (70–108)	92 (82–110)	1.017 (0.968–1.069); 0.500
Systolic blood pressure (mmHg)	Mean (range)	119 (91–143)	121 (92–143)	116 (91–143)	1.024 (0.989–1.061); 0.186
Diastolic blood pressure (mmHg)	Mean (range)	77 (61–95)	78 (64–95)	76 (61–79)	1.060 (0.991–1.133); 0.091
NYHA Class I and II	*N* (%)	9 (45)	4 (31)	5 (71)	
NYHA Class III and IV	*N* (%)	11 (55)	9 (69)	2 (29)	2.23 (0.67–7.39); 0.186

Characteristic		All patients (*N* = 20)	Deceased (*N* = 13)	Alive (*N* = 7)	Hazard ratio of death (95% CI); *P*-value
Six-minute walk test Distance (m)	Median (range)	279 (0–640)	67 (0–516)	322 (270–640)	**0.997 (0.993–0.999); 0.048**
High-sensitivity troponin T (μg/L)	Median (range)	0.13 (0.05–0.37)	0.13 (0.05–0.28)	0.13 (0.07–0.37)	1.089 (0.004–324.60); 0.977
NT-proBNP (pMol/L)	Median (range)	1410 (296–6600)	1712 (473–6600)	552 (296–3321)	1.000 (0.999–1.001); 0.081
Amyloidogenic serum FLC concentration, dFLC (mg/L)	Median (range)	342 (34–6544)	463 (65–6544)	177 (34–2322)	1.000 (0.999–1.000); 0.158
Updated Mayo Clinic disease stage					
I and II	Number (%)	0	0	0	
III	*N* (%)	7 (35)	3 (23)	4 (57))	1.000
IV	*N* (%)	13 (65)	10 (77)	3 (43)	1.987 (0.545–7.240); 0.298

Characteristic		All patients (*N* = 20)	Deceased (*N* = 13)	Alive (*N* = 7)	Hazard ratio of death (95% CI); *P*-value
Number of organs involved by amyloid	Median (range)	2 (1–6)	3 (2–6)	2 (1–5)	1.060 (0.775–1.449); 0.713
Anti-arrhythmic therapy					
β-Blocker	*N* (%)	6 (30)	5 (38)	1 (14)	NA
Amiodarone	*N* (%)	1 (5)	1 (8)	0	NA
Flecainide (prn basis)	*N* (%)	1 (5)	0	1 (14)	NA
Renin–angiotensin system blockade	*N* (%)	10 (50)	6 (46)	4 (57)	NA
Diuretic therapy					
Loop diuretic	*N*(mg/day median)	17 (40)	11 (50)	6 (35)	NA

Characteristic		All patients (*N* = 20)	Deceased (*N* = 13)	Alive (*N* = 7)	Hazard ratio of death (95% CI); *P*-value
Mineralocorticoid receptor antagonists	*N*(mg/day median)	2 (50)	2 (50)	0	NA
Sinus rhythm	*N* (%)	18 (90)	11 (85)	7 (100)	NA
Atrial fibrillation/flutter	*N* (%)	2 (10)	2 (15)	0	NA
PR interval (ms)	Median (range)	200 (80–272)	180 (80–272)	202 (120–248)	0.998 (0.984–1.011); 0.755
QRS duration (ms)	Median (range)	102 (86–130)	102 (86–130)	98 (90–106)	1.042 (0.999–1.086); 0.054
QTc duration (ms)	Median (range)	455 (379–518)	458 (379–518)	449 (401–457)	1.018 (0.999–1.038); 0.069
LV ejection fraction by biplane simpson (%)	Median (range)	42 (28–60)	38 (28–60)	52 (37–60)	0.014 (0.000–8.699); 0.193
Septal wall thickness (cm)	Median (range)	1.5 (1.2–1.9)	1.5 (1.2–1.9)	1.5 (1.2–1.8)	0.743 (0.047–11.647); 0.832

Characteristic		All patients (*N* = 20)	Deceased (*N* = 13)	Alive (*N* = 7)	Hazard ratio of death (95% CI); *P*-value
***E*/*E′* (diastolic function)**	Median (range)	19.9 (13.5–33.4)	22.4 (17.0–33.4)	17.7 (13.5–29.3)	1.053 (1.000–1.109); 0.050
Global strain by tissue Doppler imaging (%)	Median (range)	−10.3 (−4.3 to −14.0)	−7.9 (−4.3 to −12.3)	−11.9 (−9.8 to −14.0)	**0.799 (0.653–0.978); 0.029**

LV, left ventricle; *E*/*E′*, ratio of mitral peak velocity of early filling (*E*) to early diastolic mitral annular velocity (*E′*); dFLC, difference between amyloidogenic and uninvolved serum free light chain; NYHA, New York Heart Association; PR, QRS, and QT, relevant intervals on electrocardiogram; NA, not applicable; HR, hazard ratio (expressed per unit increase in variable).

Significant differences are shown in bold.

Eighteen patients, including one who was known to have paroxysmal atrial fibrillation, were in sinus rhythm (SR), one was in atrial flutter with variable AV block and one had atrial fibrillation. The PR interval was normal at baseline among 8 of 18 patients, first degree heart block was present in 9 of 18 cases, and one patient had Mobitz type 1 second degree AV block (Wenckebach phenomenon). There was intraventricular conduction delay in 11/20 patients although none had complete bundle branch block, defined as QRS duration >0.12 seconds, at baseline. Echocardiograms showed moderate and severe diastolic dysfunction among 12 of 20 and 8 of 20 patients, respectively. Median E/E′ and E/A wave ratios were 19.9 and 2.38, respectively, and median deceleration time was 145 ms. Median (range) global LV strain was −10.3% (−4.3 to −14.0) and median (range) difference between apical and basal strain was −9.5% (−3.7 to −29.3).

Estimated median follow-up time from ILR placement was 308 days (range 10–399). All patients received chemotherapy as treatment for their systemic AL amyloidosis, commenced a median of 16 days after insertion of the ILR. Most received cyclophosphamide, bortezomib (Velcade), and dexamethasone (CVD) chemotherapy and the bortezomib and cyclophosphamide in each case were administered once per week at doses of 0.7–1.0 mg/m^2 and 250–500 mg respectively; steroid regimens and doses varied substantially (table 6.2).

Table 6.2 Drug therapy including chemotherapy regimen, cardiac events, and outcome

Pt	Baseline rhythm	Nature of cardiac rhythm change	Anti-arrhythmic medication prior to onset of cardiac rhythm change (or at censor[a])	Chemotherapy regimen (days from last dose to cardiac rhythm change)	Intervention	Outcome (days from ILR to death/ censor)
1	SR	Sinus bradycardia (HR 25–30 bpm), significant pauses followed by CAVB	None	CVD (2)	None (NFR)	Died, 12
2	SR	Bradycardia (HR 30 bpm)	Bisoprolol 5 mg od	CVD (3)	None	Died, 22
3	SR, 1st degree HB	CAVB with significant pauses (HR 20 bpm)	None	Lenalidomide/dexamethasone (14)	CPR, TP	Died, 57
4	SR, 1st degree HB	CAVB (HR 25)	None	CVD (4)	CPR, PPM	Died, 10
5	Atrial flutter	CAVB (HR 20 bpm)	Amiodarone 200 mg od	CVD (3)	None	Died, 44
6	SR	Sinus bradycardia (HR 35 bpm)	Bisoprolol 3.75 mg od	CVD (7)	CPR	Died, 34

7	SR	None detected up to 4 days prior to death	None	CVD (3)	None	Died, 75
8	SR	CAVB (HR 30 bpm)	Bisoprolol 1.25 mg od	CVD (4)	CPR, TP	Died, 17
9	SR, 1st degree HB	Atrial flutter 120–160 bpm	None	CVD (4)	Atrial ablation	Alive, 399
10	SR	AF at >140 bpm	None	CVD (NA)	Amiodarone	Alive, 308
11	SR	None detected	None[a]	CVD (NA)	NA	Alive, 353
12	SR, 1st degree HB	None detected	None[a]	CVD (NA)	NA	Alive, 357
13	SR, 1st degree HB	Sinus bradycardia, significant pauses and CAVB	None	None (CVD later)	PPM	Alive, 300

14	SR, Wenckebach	CAVB (30 bpm) followed by NSVT	Bisoprolol 1.25 mg od	CVD (6)	None	Died, 45
15	SR	None detected up to 6 days prior to death	None	CVD (6)	None	Died, 36
16	SR, 1st degree HB	None detected	Bisoprolol 1.25 mg od	Melphalan/dexamethasone (NA)	NA	Died, 81
17	SR, 1st degree HB	None detected	Bisoprolol 1.25 mg od	CVD (12)	NA	Died, 65
18	SR, 1st degree HB Paroxysmal AF	AF	None	CVD (NA)	Amiodarone	Alive, 186
19	SR, 1st degree HB	AF at 140 bpm	None	CVD (1)	Bisoprolol	Alive, 193
20	AF	None detected	None	CVD (NA)	NA	Died, 49

SR, sinus rhythm; CVD, cyclophophosphamide, velcade, and dexamethasone; HB, heart block; CAVB, complete atrioventricular block; TP, temporary pacing; PPM, permanent pacemaker; CPR, cardiopulmonary resuscitation; AF, atrial fibrillation; NK, not known; ChemoRx, chemotherapy treatment; od, once daily.

[a] Medication at time of censor among those without a cardiac rhythm change.

Patient Deaths

Thirteen patients died and median overall survival by Kaplan-Meier analysis was 61 days. Weekly ILR recordings in all 13 patients failed to identify potentially fatal dysrhythmias during the course of treatment other than the immediate pre-cardiac arrest rhythm, identified only when the ILR was obtained posthumously (Table 6.3). Eight of 13 patients died in hospital and the terminal event in all 8 cases was PEA. Median time from onset of dysrhythmia to PEA was 1 hour (range 0.1 to 48 hours). Five patients died at home or in a hospice. The immediate pre-cardiac arrest recordings were retrieved from 8 of 13 patients and in all cases the initial change in cardiac rhythm was bradycardia ≤ 35bpm (Table 6.3), including complete atrioventricular block (CAVB) in 6 cases. Only 1 episode of rapid VT was recorded and this arose during CAVB-associated extreme bradycardia and spontaneously reverted to CAVB after 60 seconds.

The only baseline parameters associated with risk of death were global LV strain on echocardiogram [P = 0.029; HR 0.799 (CI 0.653–0.978) per unit improvement in strain] and 6 min walk distance [P = 0.048; HR 0.997 (CI 0.993–0.999) per meter] (Table 6.2).

Table 6.3. Events leading to cardiac arrest amongst patients who died

Patient	Baseline Rhythm and AV conduction	Pre-'cardiac arrest' rhythm	Hours from onset of pre-cardiac arrest rhythm to PEA	Intervention	Cardiac activity and place at time of arrest
1	SR	Sinus bradycardia (HR 25-30 bpm) with multiple pauses followed by CAVB	48	None (NFR)	PEA in hospital
2	SR	Bradycardia (HR 30 bpm)	0.5	None	PEA in hospital
3	SR 1st degree HB	CAVB and multiple pauses (HR 20 bpm)	12	CPR, TP (3 hours after development of CAVB)	PEA in hospital
4	SR 1st degree HB	CAVB (HR 25 bpm)	1.5	CPR and inotropic support, PPM (8.5 hours after development of CAVB)	PEA in hospital
5	Atrial flutter	CAVB (HR 20 bpm) with ventricular escape rhythm	0.5	None	PEA or asystole at home
6	SR	Bradycardia (HR 35 bpm)	0.1	CPR	PEA in hospital
7	SR	NK (SR up to 4 days prior to death)	NA	None	ILR recording unavailable,

					Died at home
8	SR	CAVB (HR 30 bpm)	3	CPR, inotropic support and TP (5.5 hours after development of CAVB)	PEA in hospital
14	SR (Wenckebach Phenomenon)	CAVB followed by 60 seconds of fast VT, then CAVB (HR 30 bpm) followed by asystole	1	None	PEA or asystole at home
15	SR	NK (SR up to 6 days prior to death)	NA	None	ILR recording unavailable, PEA in hospital

Abbreviations: SR – sinus rhythm; CAVB – complete atrioventricular block; NFR – not for cardiopulmonary resuscitation, PEA – pulseless electrical activity; HR – heart rate; BPM – beats per minute; TP – temporary pacing; PPM – permanent pacemaker; NA – not applicable; ILR – implantable loop recorder; CPR – cardiopulmonary resuscitation, VT – ventricular tachycardia; NK – not known. Where pauses are mentioned, these were >3 seconds

Arrhythmias

A total of 272 downloads were received during the course of the study. Interestingly, none of the symptom-driven manual downloads showed any rhythm change from baseline. Arrhythmias that were captured automatically by the ILR and medical interventions for those arrhythmias are shown in Table 6.2. Six (30%) patients had atrial arrhythmias detected at some point during the study period, including 4 patients who were in SR at baseline but developed an atrial arrhythmia during the course of follow- up. One of six such patients was rate controlled without a requirement for anti-arrhythmic medication, 3 cases were successfully rate controlled with amiodarone, one with a beta-blocker, and one patient underwent successful atrial flutter ablation. One patient who was alive at censor, developed 2:1 AV block and rapidly progressed to CAVB. He was not receiving anti-arrhythmic therapy. He maintained his blood pressure until he had a pacemaker implanted and he remained pacemaker dependent throughout six cycles of CVD chemotherapy and at censor, 300 days later.

Timing and dosage of chemotherapy in relation to onset of change in cardiac rhythm, including pre-terminal bradyarrhythmias, was variable and there was no consistent pattern to suggest that any particular chemotherapeutic agent or dosage was pro-arrhythmogenic. Among a total of 9 patients who developed severe bradycardia during the study, four were receiving beta blocker therapy and 1 was receiving amiodarone at the time. One of seven surviving patients was receiving beta blocker therapy at censor, bisoprolol 1.25 mg daily.

Discussion

This is the first study of ILR placement in a series of patients with cardiac AL amyloidosis. Mayo stage III cardiac AL amyloidosis is associated with a high rate of SCD, particularly in patients with a history of syncope, and this study confirms previous observations [30, 186].

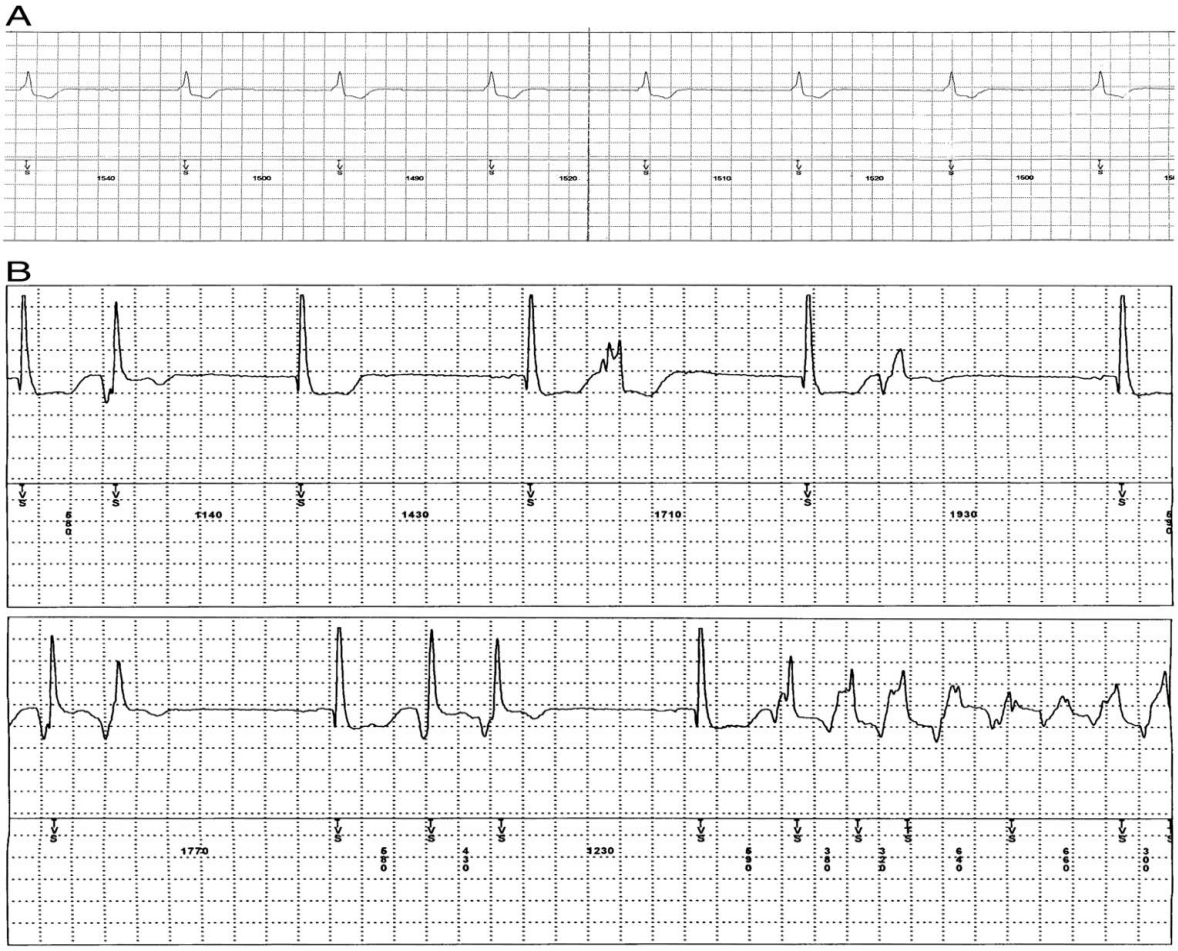
However, the surprising and completely novel finding here was that every available recording from the terminal syncopal phase prior to death showed that the initial cardiac decompensation was associated with development of marked bradycardia, usually caused by CAVB, and that despite chemotherapy with CVD, there was only one episode of non-sustained ventricular tachycardia observed throughout the whole follow up period. Our findings are consistent with previous suggestions that the terminal event in patients with cardiac AL amyloidosis is typically EMD/PEA, and also supports those who have reported that ventricular arrhythmias are uncommon in cardiac AL amyloidosis, although the data in this regard are conflicting [30, 129, 185, 186, 188]. The reasons for these discrepancies remain unclear but might be related to the use of different heart failure or chemotherapeutic agents or alternatively, related to disease severity. It is possible that ventricular tachyarrhythmias in cardiac amyloidosis are frequently self-limiting and tend to occur in patients with relatively early stage disease but that, as the disease progresses, the likelihood of AV block and consequent bradycardia increases. This hypothesis would also be consistent with the fact that ventricular tachyarrhythmias have been reported to be amenable to ICD therapy and have not generally been found to be an independent risk factor for poor prognosis in the context of AL amyloidosis [187].

We propose two hypotheses regarding the pathogenic significance of severe bradycardia in cardiac AL amyloidosis. It might simply be a pre-terminal event reflecting a very sick amyloidotic myocardium. Alternatively, however, the reduction of cardiac output associated with bradycardia might be sufficient to induce global ischaemic stunning of the heavily infiltrated ventricular myocardium, triggering irreversible decompensation and progression to PEA within a short period. This latter explanation is reminiscent of the observation that acute tubular necrosis, which is usually a reversible renal lesion which complicates an episode of renal ischaemia, rarely recovers in the context of pre-existing renal amyloidosis

however minor the ischaemic insult [313]. If ischaemic stunning is indeed the mechanism, it is likely that there may only be a very narrow time window in which to intervene among patients with cardiac AL amyloidosis who develop bradycardia. In keeping with this hypothesis, the only patient in the cohort who survived severe bradycardia had his PPM inserted before losing cardiac output, whereas three patients who received a pacemaker after resuscitation from PEA succumbed shortly thereafter. These two hypotheses could be reliably distinguished by prophylactic pacemaker insertion into patients with Mayo stage III cardiac AL amyloidosis who have pre-syncope or syncope. If interrogation of the pacemakers revealed that rapid correction of bradycardia by cardiac pacing did not prevent the terminal decline seen here, this would constitute strong evidence that bradycardias are a 'symptom' rather than a 'cause' of terminal amyloidotic myocardial failure. Interestingly, a recent study of prophylactic pacemaker insertion among patients with familial amyloid polyneuropathy, who typically have cardiac and neuropathic infiltration by amyloid composed of variant transthyretin and are at risk of conduction disorders, showed a reduction in major cardiac events over a 45-month mean follow-up among those who received pacemakers [314]. It is noteworthy that the absence of a definite survival advantage conferred by ICD implantation in individuals with cardiac AL amyloidosis does not exclude potential benefit from permanent pacemaker insertion and is entirely in keeping with our findings [185, 188]; the manufacturer's setting for the pacing capability of standard ICD devices is routinely 35 bpm, similar to the typical ventricular escape rate among patients with CAVB, and it may simply be that the reduction in cardiac output associated with this cardiac contraction rate in an individual with a small, stiff ventricle exhibiting restrictive physiology is sufficient to induce global myocardial 'stunning' thus triggering the irreversible decline highlighted above.

One important remaining question is whether chemotherapeutic agents that are administered to treat systemic AL amyloidosis may worsen prognosis among those with severe cardiac infiltration, either by promoting arrhythmias or affecting systolic function. Among this small group of severely affected individuals, nearly all of whom received combination chemotherapy with cyclophosphamide, bortezomib (Velcade) and dexamethasone (CVD), there was no indication of direct drug toxicity; arrhythmias/SCD did not occur within 48 hours of administration of chemotherapy in any patient in the series. Furthermore, one patient developed CAVB prior to receiving chemotherapy, indicating a substantial contribution to the conduction disturbance from the disease itself. Similarly, whether beta-blocker therapy should be completely avoided in patients with cardiac AL amyloidosis is not known and needs to be prospectively evaluated. Further, larger ILR studies in patients with cardiac AL amyloidosis, including those receiving a wide variety of chemotherapeutic agents as well as conventional anti-arrhythmic and cardiac failure medications, will be useful to definitively answer the question of drug toxicity in patients with severe cardiac AL amyloidosis.

Fig. 6. a) ILR recording showing severe bradycardia in a patient who proceeded to have a permanent pacemaker implanted but subsequently died. b) ILR recording showing severe bradycardia followed by onset of ventricular tachycardia.



RESULTS AND DISCUSSION

Chapter 7: Outcome of Light Chain Deposition Disease with Chemotherapy

This chapter is based on the publication below:

Natural history and outcome of light chain deposition disease.

Sayed RH, Wechalekar AD, Gilbertson JA, Bass P, Mahmood S, Sachchithanantham S, Fontana M, Patel K, Whelan CJ, Lachmann HJ, Hawkins PN, Gillmore JD.

Blood. 2015 Dec 24;126(26):2805-10

Aim

To report on the clinical presentation, histologic features, molecular basis and outcome among 53 patients with LCDD who were prospectively followed at the UK National Amyloidosis Centre, highlighting the importance of aggressively treating the underlying monoclonal proliferative disease.

Introduction

Light chain deposition disease (LCDD) is a multi-system disorder and, similar to AL amyloidosis, arises from the deposition of monotypic immunoglobulin LCs in various organs [258]. It is the most common subtype of a group of multi-system disorders characterised by the deposition of monoclonal immunoglobulin light (LCDD) or heavy chains (HCDD) or both (LHCDD) in various organs and termed monoclonal immunoglobulin deposition disease (MIDD) [258, 315, 316]. LCs are overproduced by an abnormal B- cell clone and so LCDD

is usually described in conjunction with a PCD or other lymphoproliferative disorders, most commonly multiple myeloma. However, it can occur in the absence of any detectable haematological disorder and is then called idiopathic LCDD. Clinical manifestations of LCDD vary, depending upon which organs are involved [317]. Since LCs are filtered by the glomeruli, reabsorbed in proximal tubules by receptor-mediated endocytosis, and degraded in tubular cells by lysosomal enzymes, the kidney is the principal target for LC deposition, and renal involvement and dysfunction usually dominate the clinical disease course [258, 317-320]. Liver, cardiac and neural deposits have also been documented however, and need to be considered in all newly diagnosed patients with LCDD [319, 321, 322].

LCDD typically presents with hypertension, invisible haematuria and proteinuria and, in the absence of therapy, the clinical course is one of inexorably progressive CKD leading to a requirement for RRT [315, 317, 323, 324]. Reported outcomes with renal transplantation have generally been poor, with most allograft failures occurring within a few years from recurrent LCDD; with suppression of the underlying disease, recurrence could largely be avoided and transplantation could provide good renal excretory function and so obviate the need for dialysis [325, 326]. While some studies suggest that chemotherapy may slow renal decline, data is generally limited and in the form of individual case reports [327-329].

Patients

We included in this study all 53 patients with biopsy-proven LCDD followed at the NAC between 2002 and 2015. Patients attended the NAC for their initial evaluation and were followed at regular intervals (usually six monthly) for evaluation of organ function and haematologic parameters. Attendance at the NAC included a comprehensive clinical review, serial electrocardiographic and echocardiographic evaluation, and detailed serum and urine biochemistry. No patients were lost to follow-up.

Demographic and clinical characteristics at the point of initial presentation, as well as laboratory and haematologic data, were obtained from referring centres.

All patients gave informed consent and were managed in accordance with the Declaration of Helsinki. The study was approved by the Royal Free Hospital Ethics Committee.

Materials and Methods Specific to this Study

Renal Histology, Immunohistochemistry and Electron Microscopy

All renal biopsies were processed by standard techniques for light microscopy, immunofluorescence and/or immunohistochemistry, and electron microscopy as has been described in the '*Material and Methods' Section'*. They were initially reported by trained pathologists locally and reviewed by the renal pathologist at the Royal Free London NHS Foundation Trust. At the NAC, the biopsies were additionally stained with Congo red dye and viewed under cross-polarised light to exclude the presence of amyloid deposits. Light microscopy was used to detect the presence of nodular glomerulosclerosis and mesangial expansion and all biopsies were examined under electron microscopy for the presence of electron dense deposits along the tubular basement membrane (TBM), glomerular basement membrane (GBM) and capillary basement membrane, a diagnostic requirement in LCDD [117, 322, 325]. All biopsies in which there was sufficient remaining material (48 of 53) were stained with antibodies to kappa and lambda immunoglobulin light chains, IgG, IgM and IgA [330].

Haematologic Investigations

All patients underwent a bone marrow biopsy to determine the plasma cell percentage and morphology. The detection and characterisation of serum and urine monoclonal immunoglobulins was performed using conventional immunoelectrophoresis and immunofixation. All patients additionally were tested for evidence of hypercalcemia, anaemia and osteolytic lesions [331, 332]. Patients were defined as having a renal lesion associated with monoclonal gammopathy of renal significance (MGRS) where the serum monoclonal protein was <30g/L and bone marrow plasmacytosis was <10% in the absence of hypercalcaemia, anaemia or osteolytic lesions [333].

Data on serum FLCs was not available for all patients at the time of biopsy as nephelometric measurements of serum FLCs (Binding Site, Birmingham, UK) only became available after 2002. In such cases, FLCs were obtained from stored serum samples.

Table 7.1. Haematologic response criteria

Response	**Criteria**
Complete Response	The normalisation of κ and λ ratio No detectable monoclonal protein by serum and urine immunofixation electrophoresis
Very Good Partial Response	Decrease in dFLC to <40 mg/l
Partial Response	>50% decrease in dFLC
No Response	≤50% decrease in dFLC

ACR is achieved with normalisation of the κ and λ ratio and in the absence of a detectable monoclonal protein by serum and urine immunofixation electrophoresis, a VGPR occurs with a decrease in dFLC to <40 mg/l, a PR with>50% decrease in dFLC and no response with ≤50% decrease in dFLC [334].

During follow up, patients were asked to provide monthly or two monthly serum samples for the measurement of FLCs and paraproteins, to allow the monitoring of the underlying clonal disorder. A haematologic response assessment was performed at 6 months from the start of chemotherapy.

Renal Investigations

Proteinuria was measured from a 24 hr urine collection at presentation and 6-monthly or annually during follow up. Glomerular filtration rate was estimated (eGFR) on the basis of the MDRD formula. Nephrotic range proteinuria (NRP) was defined as 24 hour proteinuria ≥3 g and nephrotic syndrome was NRP accompanied by serum albumin of <2.5 g/dL and peripheral oedema. Hypertension was defined by systolic BP >140 mmHg or diastolic BP >90 mmHg or a requirement for antihypertensive medication.

Renal course was determined by change in eGFR throughout follow up. Patients who were on RRT prior to receiving chemotherapy (n=19) and those whose renal course was not determined after 6 months (n=2) were excluded from the analysis of renal response in relation to haematologic response, leaving 32 evaluable patients.

Extra-Renal Investigations

All patients were rigorously and repeatedly assessed for the presence of extra-renal involvement by LCDD. Investigations included a standardised 6-minute walk test to monitor

changes in exercise tolerance, detailed echocardiography, and serology for cardiac (Nt-proBNP and High sensitivity Troponin T), renal (creatinine, GFR), liver (albumin, alkaline phosphatase, alanine aminotransferase, gamma glutamyl transferase), bone (alkaline phosphatase, corrected calcium, phosphate) and haematological (coagulation profile, protein electrophoresis and free light chain levels) dysfunction. When cardiac involvement by LCDD was suspected on the basis of biomarkers and/or echocardiography, cardiac magnetic resonance imaging and, where possible, endomyocardial biopsies were performed.

In a single case of suspected peripheral neuropathy due to light chain deposition, this was discounted following nerve biopsies. Liver LCDD was confirmed histologically in both cases in which it was suspected biochemically and lung LCDD was confirmed by surgical lung biopsy in a patient with panacinar emphysema, as previously described [335].

Statistical Methods

Renal outcomes (native and transplant) were censored at date of last GFR measurement either from the local treatment centre or from the last NAC evaluation, whichever was later. Patient survival was censored on 30th September 2014. Kaplan-Meier analyses were used to investigate both renal and patient survival and the log-rank test was used to compare differences in stratified survival analyses. Univariate analysis was used to investigate the association of histological features with GFR and proteinuria. Mann-Whitney U tests were used to compare outcomes between patients with different categories of haematological response. Median follow-up time was calculated using the reverse Kaplan-Meier method.

Study Limitations

The diversity of local chemotherapy regimens and the small numbers in each treatment group, made analysis between each regimen difficult and therefore treatment response was compared broadly between bortezomib- and non-bortezomib based regimens.

RESULTS

Patients

Fifty-one of 53 patients were diagnosed with LCDD on renal histology. Two patients were diagnosed on liver biopsy; in each case there was established proteinuric CKD but a renal biopsy had not been performed. The mean age at presentation was 56.3 years (range 29 to 78 years) and male/female ratio was 2.3:1. Patient demographics and clinical characteristics at the time of diagnosis are shown in Table 7.2. At diagnosis, 94% of patients were hypertensive and 92% had haematuria. The mean 24 hr urinary protein loss was 4.1 g (range 0.1 to 15.5 g/24hr). Ten patients received RRT before their diagnostic biopsy, including 1 patient who was diagnosed with LCDD on a renal transplant biopsy, performed to investigate deteriorating allograft function. Details of the underlying clonal dyscrasia and light chain isotype are included in Table 7.2.

Table 7.2. Demographic, clinical and haematological characteristics at diagnosis

Characteristic	**Value**
Median (range) age (years)	56.3 (29-78)
Gender (male/female) (n)	37/16
Race (n)	
White	51
Black	1
Asian	1
Hypertension (n)	50 (94%)
GFR	
CKD Stage 1	0
CKD stage 2/3	23 (43%)
CKD stage 4/5 (including RRT)	30 (57%)
Mean eGFR (ml/min)[a]	27 (0-79)
RRT before diagnosis (n):	
Dialysis	9 (17%)
Transplant	1 (2%)
Mean(range) urinary protein loss (g/24hr)	4.1 (0.1-15.5)
Nephrotic range proteinuria (n[b])	24/45 (53%)
Nephrotic syndrome (n[b])	10/45 (22%)
Microscopic haematuria (n[c])	45/50 (90%)
Haematological findings	
Frequency of Abnormal FLC levels	
LC isotype	100%
Kappa	43/53 (81%)
Lambda	10/53 (19%)

Median (range) Pathogenic FLC Value (mg/L)	
Kappa	724 (25.6-25700)
Lambda	147 (29.8-742)
Median (range) FLC ratio κ:λ (mg/L)	
Kappa	29.2 (2.9-150.8)
Lambda	0.17 (0.05-0.2)
BJP by UPEP/UIFE[b] (n)	20 (44.4%)
Monoclonal protein in serum detected by immunofixation (n)	23 (43.4%)
No monoclonal protein in serum detected by immunofixation (n)	19 (35.8%)
Extra-renal Involvement	5 (9.4%)
Cardiac	2 (3.8%)
Hepatic	2 (3.8%)
Lung	1 (1.9%)
Neuropathy	0 (0%)
Underlying Clonal Cell Disorder	
PCD	52 (98%)
≥10% plasma cells	*6 (11.3%)*
<10% plasma cells	*46 (86.8%)*
CLL (n/N)	1 (2%)

BJP, Bence Jones Protein; CKD, chronic kidney disease; CLL, chronic lymphocytic leukaemia. LC, light chain MGRS, monoclonal gammopathy of renal significance; RRT, renal replacement therapy; UPEP/UIFE, urine protein electrophoresis/urine immunofixation electrophoresis. [a]-an eGFR of 0 was used for patients on renal replacement therapy, [b] - no 24-hour urine collection performed among 8 of those receiving dialysis at the time of renal biopsy, [c] -- no documentation of presence/absence of haematuria in 3 patients.

Histology

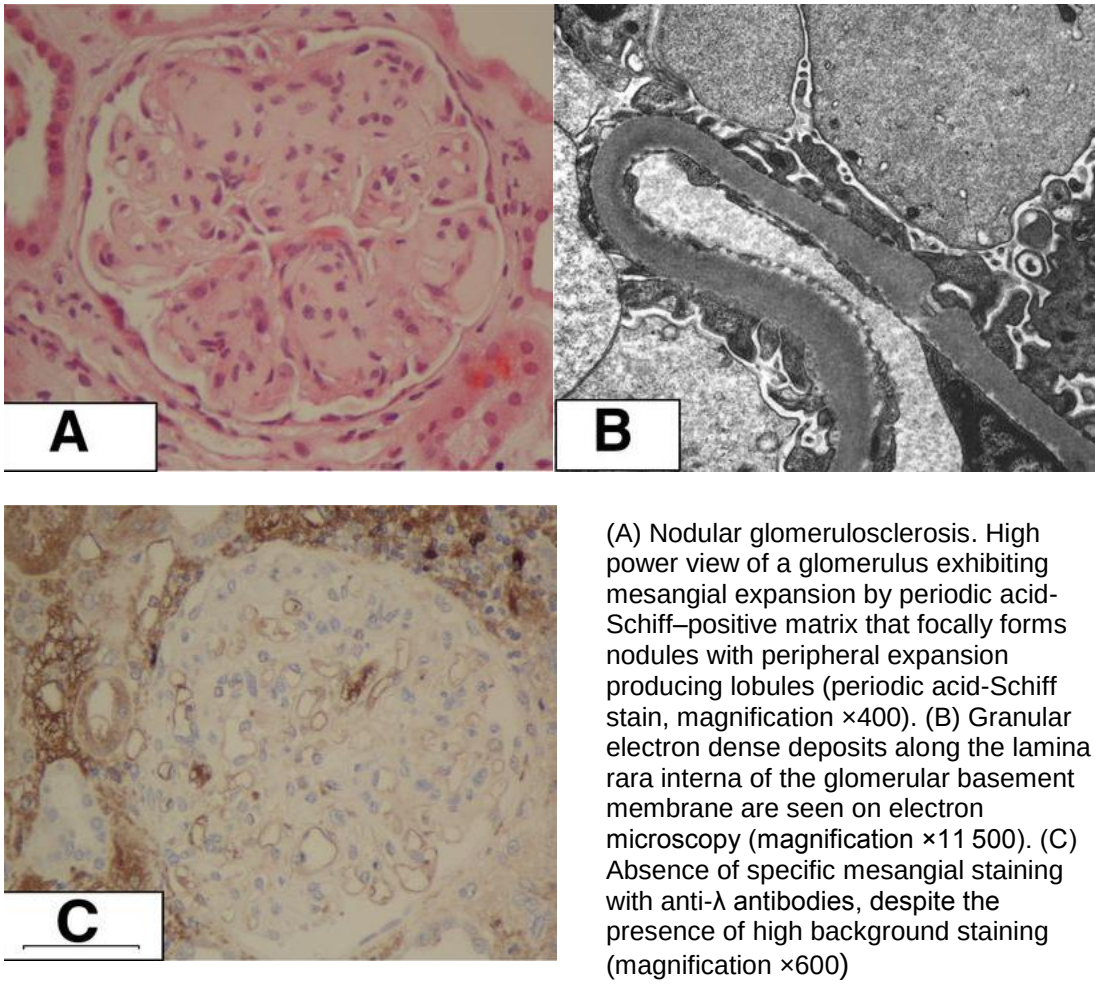
Renal biopsy findings are detailed in Table 7.3.

Table 7.3 Renal biopsy histology

	Number of Patients (N=51)	Percentage Patients
Light microscopy		
Nodular mesangial sclerosis	29	56.9
Interstitial fibrosis	31	60.8
Tubular atrophy	43	84.3
Mesangial expansion	25	49.0
Immunofluorescence microscopy		
Specific staining with 'causative' light chain*	24	47.0
Non-specific staining with κ and λ	2	3.9
Absence of staining with either κ or λ	20	39.2
Insufficient material	5	9.8
Electron microscopy		
Electron dense deposits	51	100
GBM	34	66.7
TBM	20	39.2
Mesangial	23	45.1
Vascular	6	11.8
Subepithelial	2	3.9
Subendothelial	15	29.4

On LM, nodular mesangial sclerosis (Figure 7.1A) was present in 56% of patients, mesangial expansion in 49%, tubular atrophy in 84%, and interstitial fibrosis in 61%. Amyloid deposits were excluded by Congo red staining in all biopsies. Nearly one third of cases failed to stain specifically with antibodies against kappa or lambda immunoglobulin LCs (Figure 7.1B, 7.1C), although in every such case there was unequivocal evidence of a clonal proliferative disease by sFLC assay, typical LM findings, and characteristic granular electron-dense deposits along basement membranes by EM. Indeed, granular electron-dense deposits were identified by EM in all renal biopsies, as well as in the 2 respective liver specimens.

Fig. 7.1 Histology in LCDD



(A) Nodular glomerulosclerosis. High power view of a glomerulus exhibiting mesangial expansion by periodic acid-Schiff–positive matrix that focally forms nodules with peripheral expansion producing lobules (periodic acid-Schiff stain, magnification ×400). (B) Granular electron dense deposits along the lamina rara interna of the glomerular basement membrane are seen on electron microscopy (magnification ×11 500). (C) Absence of specific mesangial staining with anti-λ antibodies, despite the presence of high background staining (magnification ×600)

None of the histological features were significantly associated with either baseline GFR or degree of proteinuria. Furthermore, neither the presence of nodular glomerulosclerosis (P=0.639) nor interstitial fibrosis (P=0.475) was significantly associated with renal survival.

Renal outcomes in relation to baseline investigations

Median follow-up for the whole cohort was 6.2 years (range, 1.1-14.0 years). Among 43 patients who were not receiving RRT at the time of diagnosis, the mean rate of GFR decline (prior to RRT or death) was 3.7 mL/min per year. Twenty-three patients commenced dialysis during follow-up; median renal survival from diagnosis by Kaplan-Meier estimate for those who were not already receiving RRT at baseline was 5.4 years (Figure 7.2A). Unsurprisingly, CKD stage at diagnosis had a significant impact on renal survival with those who had CKD stage 2 or 3 at diagnosis remaining dialysis independent for a median of 9.0 years compared with only 2.7 years among those with CKD stage 4 or 5 at diagnosis (excluding the 10 patients already receiving RRT at the time of biopsy; P = 0.004; Figure 7.2B). There was no significant difference in renal survival between patients who had nephrotic range proteinuria (>3 g/24 hr) at diagnosis and those with subnephrotic proteinuria, although proteinuria >6 g/24 hr was associated with reduced renal survival (P = 0.017). Renal survival was not influenced by the degree of bone marrow plasmacytosis.

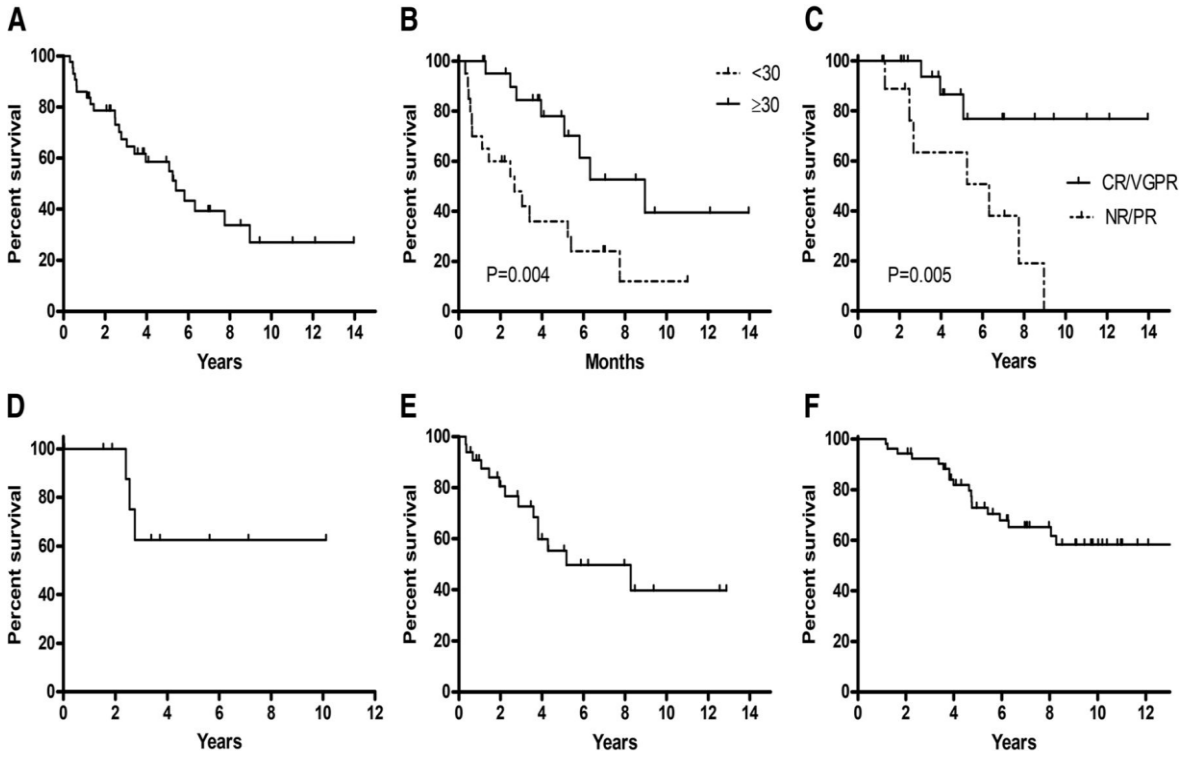


Fig 7. 2 Renal and patient survival in LCDD. (A) Median renal survival from diagnosis of LCDD was 5.4 years. (B) Renal survival was significantly longer among those who were diagnosed, whereas eGFR was ≥30 mL/min per year compared with those diagnosed once GFR was <30 mL/min per year (P = .004). (C) Renal survival was significantly longer among patients who achieved a hematologic CR/VGPR with first-line chemotherapy compared with those who achieved a hematologic PR/NR (P = .005). (D) Renal survival from ASCT among 11 patients with median GFR of 24 mL/min per year at the start of the procedure. (E) Patient survival from commencement of dialysis, censored at the time of transplantation. (F) Patient survival from diagnosis of LCDD; median estimated survival was 14.0 years.

Renal outcome in relation to haematologic response

We compared renal outcomes among 32 evaluable patients who achieved different levels of clonal response (as determined by changes in their dFLC values) to chemotherapy and/ or ASCT, where the latter was being administered to achieve a haematologic response and not to consolidate an ongoing response to chemotherapy. The chemotherapy regimens that were administered to these patients are shown in Table 7.4 and, as there are no existing guidelines as to treatment of LCDD at the time of the study, chemotherapy choice was left to the patients' local centre.

Table 7.4. Chemotherapy regimens and haematologic response

	n/32	CR	VGPR	PR	NR
Cyclophosphamide, Bortezomib, Dexamethsone (CyBorD)	4	3		1	
Cyclophosphamide, Thalidomide, Dexamethsone (CTD)	9	4	2	1	2
Vincristine, Adriamycin, Dexamethasone (VAD)	2	2			
Velcade, Dexamethasone	4	4			
Melphalan, Dexamethasone	3				3
Lenalidomide, Prednisolone	1			1	
Melphalan autologous stem cell transplant	4	4			
Thalidomide, Dexamethasone	1				1
Thalidomide, Prednisolone	1				1
Rituximab, Cyclophosphamide	1			1	
Cyclophosphamide, Dexamethasone	1	1			
No treatment	1				1

There was a mean improvement in GFR of 6.1ml/min per year from a baseline of 26 ml/min among 21 patients who achieved a haematological Complete Response (CR)/Very Good Partial Response (VGPR), compared with a mean GFR loss of 6.5 ml/min per year from a baseline of 34 ml/min among both those who achieved a Partial Response (PR) (n=4) and those who failed to achieve a haematologic response to chemotherapy, No Response (NR) (n=7) (Figure 7.3, P=0.005). Only 3 of 21 patients who achieved an initial haematologic CR/VGPR required RRT throughout follow up, compared to 6 of 11 patients who achieved an initial PR/NR (P<0.04). Median time to haematologic relapse among responders was 3.6 years.

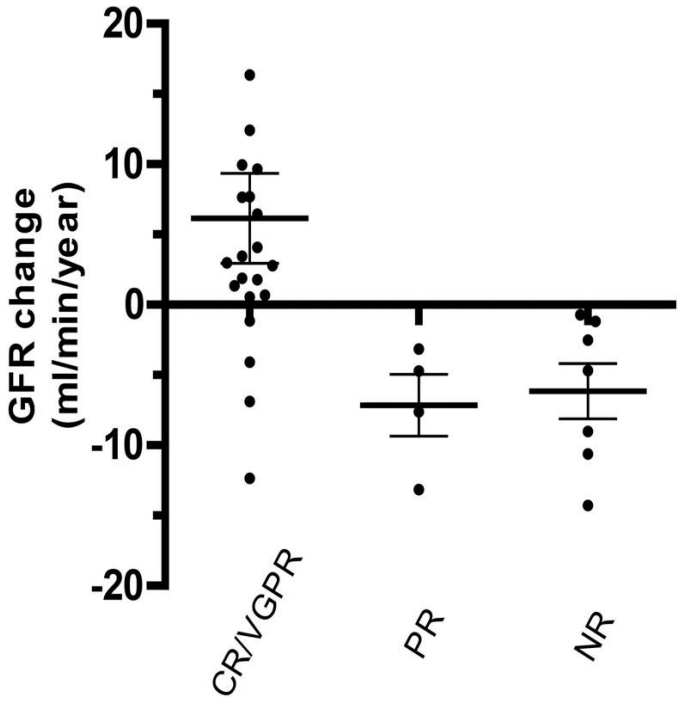


Fig. 7.3
Rate of change of GFR in relation to hematologic response. The scatter graph and plots below demonstrate the rate of change in GFR per year and the mean ± standard error of the mean for each dataset for patients with varying degrees of clonal response. GFR increased among those achieving either a hematologic CR or VGPR but declined among those who achieved only a PR or NR with chemotherapy (Mann-Whitney U test, P < .009).

Sixteen patients in the whole cohort (median age, 50 years; range, 38-72 years) received a melphalan-conditioned ASCT. Median pre-treatment bone marrow plasmacytosis among those who underwent ASCT was 12% (range, 4-50%). Four patients were dialysis

dependent prior to ASCT, the aim of which was to suppress the plasma cell dyscrasia to decrease the likelihood of disease recurrence following subsequent renal transplantation, and 1 patient had a functioning renal transplant at the time of ASCT. One patient with pre-existing severe myopathy, aged 72 years, died during ASCT from pneumonia (transplant-related mortality, 6.7%). Median GFR prior to ASCT among 11 patients not requiring RRT was 24 mL/min per year (range, 11-51 mL/min per year), which had increased among the 10 surviving patients to 38 mL/min per year (range dialysis to >90 mL/min per year) at the end of follow-up. Thirteen of 15 patients who survived ASCT achieved a hematologic CR and 2 achieved PR. After a median follow-up from ASCT of 4.4 years (range, 0.04-12.2 years), only 2 of 14 patients required further chemotherapy for haematologic relapse, administered 5.2 and 5.5 years later. Only 3 of 11 patients who were not on RRT prior to ASCT, all of whom had CKD stage 4 at the time of ASCT, eventually required dialysis 2.4, 2.6, and 2.8 years after the procedure; it is notable that all 3 patients had persistent nephrotic-range proteinuria following ASCT, whereas proteinuria fell following ASCT in all patients who remained dialysis independent. Renal survival after ASCT (censored at death) by Kaplan-Meier analysis for the cohort of 11 dialysis-independent patients is shown in Figure 7.2D; at censor, median renal survival had not been reached.

Outcomes with RRT

Dialysis

Thirty-three (62%) patients from the whole cohort required dialysis during their disease course (including 10 from before the diagnosis of LCDD). Eight patients received peritoneal dialysis and 25 received haemodialysis. Median patient survival from commencement of dialysis, censored at transplantation, was 5.2 years (Figure 7.2E), and there was no difference in survival between those commencing different dialysis modalities (P = 0.336).

Renal Transplantation

Seven patients from the whole cohort underwent renal transplantation, and in 3 of these cases, the transplant failed. Cause of renal allograft failure was biopsy-proven LCDD recurrence in 2 patients and rejection secondary to noncompliance with immunosuppression in the remaining patient. Neither patient whose LCDD recurred had received chemotherapy prior to renal allograft failure, which occurred after 1.6 and 1.9 years. The 4 remaining patients had GFRs of 45, 44, 50, and 50 mL/min per year at censor 9.7, 6.2, 5.1, and 0.8 years after renal transplantation, respectively, without clinical, biochemical, or histologic evidence of LCDD in their allografts. One of these patients underwent a high-dose melphalan ASCT shortly after renal transplantation without loss of GFR during the procedure, and the other 3 patients received an ASCT prior to renal transplantation. The latter 3 patients remained in complete hematologic remission at censor 9.6, 8.6, and 6.8 years after ASCT.

Extra-Renal Outcomes

There was improvement in cardiac (2 patients) and hepatic (2 patients) function among those with extra-renal LCDD who achieved a haematologic response. NT-proBNP values fell from 909 to 6 pmol/L and from 1714 to 296 pmol/L. in association with an improvement in diastolic function on echocardiography in both patients with cardiac infiltration. Alkaline phosphatase levels fell from 276 to 71 iu/L and from 522 to 159 iu/L in both patients with hepatic LCDD. One patient with pulmonary LCDD who achieved a haematologic CR has had stable spirometry but has failed to improve clinically and was awaiting lung reduction surgery at the time of censor.

Patient survival

Median estimated patient survival from diagnosis by Kaplan-Meier analysis for the whole cohort was 14.0 years (Figure 7.2F). Nineteen patients died during follow-up, and causes of death were infection (n = 6), ischaemic heart disease (n = 4), ESRD (n = 3), congestive cardiac failure (n = 2), cerebrovascular accident (n = 1), gastrointestinal haemorrhage (n = 1) and multiple myeloma (n = 2).

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Discussion

The prolonged follow-up of this large cohort of patients with LCDD highlights systematically for the first time the relationship between the response of the underlying haematologic disease to chemotherapy and renal outcome in this disease. The rate of native GFR loss, among those who did not achieve either a CR or VGPR haematologic response with chemotherapy, was 6.5 ml/min per year. This is markedly higher than that reported by Davies et al., where the average decline in GFR was 0.8 and 1.4 ml/min/yr for women and men >66 years old, respectively [336]. This is not surprising since these patients were continuing to deposit light chains in their kidneys. On the other hand, those who achieved a haematologic CR or VGPR with chemotherapy, had an improvement in GFR of 6.1 ml/min per year throughout the time that their clonal response was sustained and only 3 of 21 such cases required dialysis during follow up, despite a median GFR at the start of chemotherapy of only 26 ml/min per year. These data clearly indicat that one should strive to achieve a haematologic CR when treating patients with LCDD, even in the context of pre-existing stage 4 CKD. In addition, the data demonstrates that ASCT can be successful, even when undertaken at a low GFR (median 24 ml/min, range 11-51 ml/min in this cohort), apparently with little risk of precipitating a renal decline. Unsurprisingly however, patients treated whilst their GFR was >30 ml/min had a substantially better renal survival (median nearly 9 years) compared to patients who started treatment having already reached CKD stage 4. These

findings highlight important differences between LCDD and the more commonly diagnosed prototypic disease of monoclonal light chain deposition, renal AL amyloidosis. In renal AL amyloidosis, which is treated with chemotherapy in a similar fashion to LCDD, it is unusual to see improvements in GFR once CKD stage 4 has supervened, even in the context of a complete haematologic response [282]. Furthermore, ASCT in renal AL amyloidosis is associated with a risk of transplant-related mortality and of precipitating an irreversible acute decline in GFR, both of which would appear to be lower in LCDD [337]. Both bortezomib-based regimens and melphalan conditioned ASCT were associated with high rates of CR/VGPR in this cohort. Of note, in impaired renal function the intravenous melphalan-dose is usually reduced by up to 50% to reduce the likelihood of severe myelosuppression.

In the absence of a haematologic response, renal transplants failed rapidly from recurrence of LCDD, as previously reported [325]. However, among those patients who achieved a haematologic VGPR or CR before or shortly after renal transplantation, there was no evidence of recurrent LCDD, even after prolonged follow up. These findings imply that the optimal current treatment would involve a bortezomib-based regime and/ or ASCT to suppress pathogenic free light chain levels followed by renal transplantation. Inadequate free light chain suppression and subsequent renal transplantation resulted in renal recurrence.

Extra-renal light chain deposits were only identified in 4 patients (7.5%) from this cohort, with one patient having both cardiac and liver involvement. It is noteworthy that there did not appear to be an association between light chain concentration in the serum and presence of extra-renal disease. In all such cases, the extra-renal disease course mirrored that in the kidneys following chemotherapy, such that improvements in both cardiac and renal function were observed in association with haematologic complete responses.

In conclusion, renal and patient survival, as well as tolerance of high dose chemotherapy is markedly better in LCDD than in systemic AL amyloidosis, where median patient survival from diagnosis is 35.2 months as compared to greater than 58% of patients with LCDD surviving for more than 15 years; native renal survival in AL amyloidosis from the point of diagnosis is 26.8 months as compared to 5.8 years in LCDD [282]. LCDD should be aggressively treated, even in patients who have advanced renal impairment. Achieving a very good or complete haematologic response prevents early renal allograft failure from recurrent LCDD.

Chapter 8: General Conclusions and Further Studies

The studies described in this thesis outline several novel findings concerning the diagnosis and treatment of amyloidosis and the related disorder, light chain deposition disease.

The first study was a review of current methods, in the UK of amyloid diagnosis and pitfalls that may lead to misdiagnosis, specifically misdiagnosing amyloidosis as one of the commoner proteinuric disorders i.e. minimal change disease. If a thorough panel of staining had been performed, including Congo red staining, then in 65% of cases, amyloid could correctly have been identified. Furthermore, had electron microscopy been performed in all available samples, characteristic amyloid fibrils would have been identified. The median delay before reaching the correct diagnosis was 8 months, which of course has important implications in terms of initiating the correct treatment and the prognosis, especially as 78% of patients had cardiac involvement which we now recognise as being the main prognostic determinant. In fact, by the time of diagnosis, SAP scintigraphy revealed a moderate or large total body amyloid burden in a third of patients. Such a study evaluating current UK histological practice had not previously been undertaken.

The subsequent study proceeded on from this and hoped to establish a relatively new and previously unused diagnostic method in the UK, that is proteomics, to diagnose amyloidosis and to specify the particular subtype in a sensitive manner. To this end, Fibrinogen A α-chain amyloidosis was investigated and again this had not previously been researched and a database of fibrinogen variants was initiated. The presence of amyloid was successfully identified through the presence of ‘signature’ proteins and, once sufficient variants have been identified, gene sequencing may also become redundant. Mass spectrometry and

subsequent proteomic analysis have now been established at the NAC and are used for every amyloid subtype although, as yet, this technique is in addition to and not instead of the more conventional techniques, such as IHC and genotyping. Issues that need to be overcome and investigated in further studies include limiting the presence of background contaminants, modifying the fixation process so that the subsequent cleavage and the mass of resulting peptides is not affected and modifying the rate limiting overnight trypsin digestion step. Another important issue is that proteomics remains a semi-quantitative technique and therefore larger proteins generating more peptides and therefore appearing more prominently in the list generated, are not necessarily the causative proteins.

In order to further stratify renal damage non-invasively, specifically tubular damage and perhaps interstitial fibrosis and tubular atrophy and to help prognosticate renal outcome, I investigated a biomarker that has not yet been established as a bedside test for amyloidosis. Urinary retinol binding protein, usually standardised to the urine creatinine and termed as the RBP:Creatinine Ratio that is RCR, has only been analysed very recently in amyloidosis by Li et al. Furthermore, this study was only in patients with AL amyloidosis. I found that higher RCR values are correlated with a worsening eGFR, greater degree of glucosuria, decreased tubular phosphate reabsorption and more severe tubular atrophy across subytpes generally and higher levels of LCs for AL amyloid. Although increasing proteinuria, and therefore a lower serum albumin by association, is used as a marker of worsening renal function in amyloidosis- this did not correlate with RCR values. Interestingly, some of the RCR values obtained were higher than in any previously reported studies. In terms of further studies, some of the trends observed in the less common subtypes i.e. the hereditary amyloidoses or related disorders, such as LCDD failed to become significant and therefore including a greater number of these patients in any further studies would be useful. A more recent study has found that RBP is a significant prognostic marker in LCDD [338].

Additionally, the patients included could be followed up for longer to see whether or not any prognostic values arise or if uRBP values indicate the degree of reversibility following acute on chronic kidney damage- as observed in non-amyloid patients [339].

An important theme that underlies much of the clinical care in amyloid patients and this research, is the importance of early diagnosis and treatment, primarily to reduce the burden of amyloid deposition and especially where cardiac involvement features. In order to address whether or not specific interventions i.e. the use of an implantable cardiac defibrillator, pacemaker and/ or certain medications could affect outcome once cardiac disease has been established, I undertook a prospective cohort study into the most severely affected cardiac amyloid patients. This provided an insight into the nature of arrhythmias and, in some cases, the pre-terminal events which, in all cases, was found to be asystole/ pulseless electrical activity preceded by the development of marked bradycardia. Additionally, once patients have been resuscitated from an episode of cardiac 'arrest' the insertion of a pacemaker does not seem to convey the same prognostic advantage as that inferred by pacemaker insertion prior to an episode of cardiac 'arrest' or ventricular standstill. There is no indication that patients with severe cardiac amyloid would benefit from prophylactic ICD insertion and further studies are needed to determine whether rate limiting medication, including beta-blockers, should be avoided in severe cardiac amyloid.

AL amyloidosis is the most prevalent form of amyloidosis in the developed world and it has been proposed that the light chains responsible possess some inherent amyloidogenic properties; LCDD also arises from the deposition of monotypic immunoglobulin light and/or heavy chains although these chains lack the inherent amyloidogenic property seen in amyloidosis. Treatment for LCDD is similarly through using chemotherapy although a comprehensive study of this disorder has not previously been undertaken. We found that

patients had to have achieved at least a very good partial response to chemotherapy in order for their renal function to improve and this stood true even for those patients who were already at CKD Stage 4, which differs to treatment response in patients with AL amyloidosis who were already at CKD Stage 4. However, renal outcome was better in LCDD when treatment was started with a greater renal reserve. Additionally, autologous stem cell transplant related patient mortality and irreversible renal decline was lower in LCDD than in AL amyloid, even when undertaken at CKD Stage 4. Whatever the method or chemotherapy regimen, renal transplants failed rapidly from LCDD recurrence when a haematologic response was not achieved.

To summarise, the research undertaken has: improved our understanding and need to employ best practise in order to avoid the misdiagnosis of renal amyloid, helped establish the use of proteomics in diagnosing amyloidosis and its subtypes in the UK for the first time, explored the value of urinary biomarkers in amyloidosis, investigated arrhythmias and, in particular, pre-terminal events and provided a comprehensive review of a previously understudied related disease.

References

1. Aterman, K., *A historical note on the iodine-sulphuric acid reaction of amyloid.* Histochemistry, 1976. **49**(2): p. 131-43.
2. Sipe, J.D., et al., *Amyloid fibril protein nomenclature: 2012 recommendations from the Nomenclature Committee of the International Society of Amyloidosis.* Amyloid, 2012. **19**(4): p. 167-70.
3. Puchtler, H. and F. Sweat, *Amidoblack as a stain for hemoglobin.* Arch Pathol, 1962. **73**: p. 245-9.
4. Westermark, G.T., K.H. Johnson, and P. Westermark, *Staining methods for identification of amyloid in tissue.* Methods Enzymol, 1999. **309**: p. 3-25.
5. Picken, M.M., *New insights into systemic amyloidosis: the importance of diagnosis of specific type.* Curr Opin Nephrol Hypertens, 2007. **16**(3): p. 196-203.
6. Pepys, M.B., et al., *Binding of serum amyloid P-component (SAP) by amyloid fibrils.* Clin Exp Immunol, 1979. **38**(2): p. 284-93.
7. Richards, D.B., et al., *Therapeutic Clearance of Amyloid by Antibodies to Serum Amyloid P Component.* N Engl J Med, 2015. **373**(12): p. 1106-14.
8. Pinney, J.H., et al., *Systemic amyloidosis in England: an epidemiological study.* Br J Haematol, 2013. **161**(4): p. 525-32.
9. Wechalekar, A.D., P.N. Hawkins, and J.D. Gillmore, *Perspectives in treatment of AL amyloidosis.* Br J Haematol, 2008. **140**(4): p. 365-77.
10. Tang, W., et al., *End-stage renal failure due to amyloidosis: outcomes in 490 ANZDATA registry cases.* Nephrol Dial Transplant, 2013. **28**(2): p. 455-61.
11. Leung, N., S.H. Nasr, and S. Sethi, *How I treat amyloidosis: the importance of accurate diagnosis and amyloid typing.* Blood, 2012. **120**(16): p. 3206-13.
12. Ihse, E., et al., *Amyloid fibril composition is related to the phenotype of hereditary transthyretin V30M amyloidosis.* J Pathol, 2008. **216**(2): p. 253-61.
13. Pepys, M.B., *Amyloidosis.* Annu Rev Med, 2006. **57**: p. 223-41.
14. Bhat, A., et al., *Currents concepts on the immunopathology of amyloidosis.* Clin Rev Allergy Immunol, 2010. **38**(2-3): p. 97-106.
15. Campioni, S., et al., *A causative link between the structure of aberrant protein oligomers and their toxicity.* Nat Chem Biol, 2010. **6**(2): p. 140-7.
16. Kumar, V., R.S. Cotran, and S.L. Robbins, *Robbins basic pathology*. 7th ed ed. 2003, Philadelphia: Saunders. xii, 873 p.
17. McCubbin, W.D., et al., *Circular-dichroism studies on two murine serum amyloid A proteins.* Biochem J, 1988. **256**(3): p. 775-83.
18. Kelly, J.W., *The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways.* Curr Opin Struct Biol, 1998. **8**(1): p. 101-6.
19. Skoulakis, S. and J.M. Goodfellow, *The pH-dependent stability of wild-type and mutant transthyretin oligomers.* Biophys J, 2003. **84**(5): p. 2795-804.
20. Zhao, L., J.N. Buxbaum, and N. Reixach, *Age-Related Oxidative Modifications of Transthyretin Modulate Its Amyloidogenicity.* Biochemistry, 2013. **52**(11): p. 1913-1926.
21. Lee, C.C., et al., *A three-stage kinetic model of amyloid fibrillation.* Biophys J, 2007. **92**(10): p. 3448-58.
22. Merlini, G. and V. Bellotti, *Molecular mechanisms of amyloidosis.* N Engl J Med, 2003. **349**(6): p. 583-96.

23. Bodin, K., et al., *Antibodies to human serum amyloid P component eliminate visceral amyloid deposits.* Nature, 2010. **468**(7320): p. 93-7.
24. Lundmark, K., A. Vahdat Shariatpanahi, and G.T. Westermark, *Depletion of spleen macrophages delays AA amyloid development: a study performed in the rapid mouse model of AA amyloidosis.* PLoS One, 2013. **8**(11): p. e79104.
25. Merlini, G. and M.J. Stone, *Dangerous small B-cell clones.* Blood, 2006. **108**(8): p. 2520-30.
26. Nasr, S.H., et al., *The diagnosis and characteristics of renal heavy-chain and heavy/light-chain amyloidosis and their comparison with renal light-chain amyloidosis.* Kidney Int, 2013. **83**(3): p. 463-70.
27. Brumshtein, B., et al., *Formation of amyloid fibers by monomeric light chain variable domains.* J Biol Chem, 2014. **289**(40): p. 27513-25.
28. Falk, R.H., R.L. Comenzo, and M. Skinner, *The systemic amyloidoses.* N Engl J Med, 1997. **337**(13): p. 898-909.
29. Merlini, G., D.C. Seldin, and M.A. Gertz, *Amyloidosis: pathogenesis and new therapeutic options.* J Clin Oncol, 2011. **29**(14): p. 1924-33.
30. Dubrey, S.W., et al., *The clinical features of immunoglobulin light-chain (AL) amyloidosis with heart involvement.* QJM, 1998. **91**(2): p. 141-57.
31. Dember, L.M., *Amyloidosis-associated kidney disease.* J Am Soc Nephrol, 2006. **17**(12): p. 3458-71.
32. Palladini, G., P. Milani, and G. Merlini, *Novel strategies for the diagnosis and treatment of cardiac amyloidosis.* Expert Rev Cardiovasc Ther, 2015. **13**(11): p. 1195-211.
33. van der Hilst, J.C., *Recent insights into the pathogenesis of type AA amyloidosis.* ScientificWorldJournal, 2011. **11**: p. 641-50.
34. Benditt, E.P., N. Eriksen, and R.H. Hanson, *Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein.* Proc Natl Acad Sci U S A, 1979. **76**(8): p. 4092-6.
35. Malle, E. and F.C. De Beer, *Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice.* Eur J Clin Invest, 1996. **26**(6): p. 427-35.
36. Ray, A., et al., *Inflammation-responsive transcription factor SAF-1 activity is linked to the development of amyloid A amyloidosis.* J Immunol, 2006. **177**(4): p. 2601-9.
37. Ganapathi, M.K., et al., *Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells.* J Immunol, 1991. **147**(4): p. 1261-5.
38. Migita, K., et al., *Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro.* FEBS Lett, 2004. **569**(1-3): p. 235-9.
39. Gollaher, C.J. and L.L. Bausserman, *Hepatic catabolism of serum amyloid A during an acute phase response and chronic inflammation.* Proc Soc Exp Biol Med, 1990. **194**(3): p. 245-50.
40. Lachmann, H.J., et al., *Natural history and outcome in systemic AA amyloidosis.* N Engl J Med, 2007. **356**(23): p. 2361-71.
41. Sattianayagam, P.T. and P.N. Hawkins, *Systemic Amyloidosis*, in *Textbook of Clinical Gastroenterology and Hepatology*, H. C.J., et al., Editors. 2012, Wiley-Blackwel: Oxford.
42. Gillmore, J.D., et al., *Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein.* Lancet, 2001. **358**(9275): p. 24-9.
43. Hund, E., *Familial amyloidotic polyneuropathy: current and emerging treatment options for transthyretin-mediated amyloidosis.* Appl Clin Genet, 2012. **5**: p. 37-41.
44. Galat, A., et al., *Aortic stenosis and transthyretin cardiac amyloidosis: the chicken or the egg?* Eur Heart J, 2016. **37**(47): p. 3525-3531.
45. Rapezzi, C., et al., *Cardiac amyloidosis: the great pretender.* Heart Fail Rev, 2015. **20**(2): p. 117-24.

46. Gonzalez-Lopez, E., et al., *Wild-type transthyretin amyloidosis as a cause of heart failure with preserved ejection fraction.* Eur Heart J, 2015. **36**(38): p. 2585-94.
47. Ng, B., et al., *Senile systemic amyloidosis presenting with heart failure: a comparison with light chain-associated amyloidosis.* Arch Intern Med, 2005. **165**(12): p. 1425-9.
48. Tanskanen, M., et al., *Senile systemic amyloidosis affects 25% of the very aged and associates with genetic variation in alpha2-macroglobulin and tau: a population-based autopsy study.* Ann Med, 2008. **40**(3): p. 232-9.
49. Dubrey, S.W., *Amyloid heart disease: a brief review of treatment options.* Postgrad Med J, 2012. **88**(1046): p. 700-5.
50. Dubrey, S.W. and R.H. Falk, *Amyloid heart disease.* Br J Hosp Med (Lond), 2010. **71**(2): p. 76-82.
51. Pinney, J.H., et al., *Senile systemic amyloidosis: clinical features at presentation and outcome.* J Am Heart Assoc, 2013. **2**(2): p. e000098.
52. Westermark, P., et al., *Transthyretin-derived senile systemic amyloidosis: clinicopathologic and structural considerations.* Amyloid, 2003. **10 Suppl 1**: p. 48-54.
53. Rapezzi, C., et al., *Systemic cardiac amyloidoses: disease profiles and clinical courses of the 3 main types.* Circulation, 2009. **120**(13): p. 1203-12.
54. Falk, R.H. and S.W. Dubrey, *Amyloid heart disease.* Prog Cardiovasc Dis, 2010. **52**(4): p. 347-61.
55. Hutt, D.F., et al., *Utility and limitations of 3,3-diphosphono-1,2-propanodicarboxylic acid scintigraphy in systemic amyloidosis.* Eur Heart J Cardiovasc Imaging, 2014. **15**(11): p. 1289-98.
56. Kyle, R.A., et al., *Prevalence of monoclonal gammopathy of undetermined significance.* N Engl J Med, 2006. **354**(13): p. 1362-9.
57. Hawkins, P.N., et al., *Evolving landscape in the management of transthyretin amyloidosis.* Ann Med, 2015. **47**(8): p. 625-38.
58. Ruberg, F.L. and J.L. Berk, *Transthyretin (TTR) cardiac amyloidosis.* Circulation, 2012. **126**(10): p. 1286-300.
59. Ruberg, F.L., et al., *Prospective evaluation of the morbidity and mortality of wild-type and V122I mutant transthyretin amyloid cardiomyopathy: the Transthyretin Amyloidosis Cardiac Study (TRACS).* Am Heart J, 2012. **164**(2): p. 222-228 e1.
60. Connors, L.H., et al., *Heart Failure Resulting From Age-Related Cardiac Amyloid Disease Associated With Wild-Type Transthyretin: A Prospective, Observational Cohort Study.* Circulation, 2016. **133**(3): p. 282-90.
61. Connors, L.H., et al., *Tabulation of human transthyretin (TTR) variants, 2003.* Amyloid, 2003. **10**(3): p. 160-84.
62. Schmidt, H.H.J., *Tafamidis for the treatment of transthyretin-associated familial amyloid polyneuropathy.* Expert Opinion on Orphan Drugs, 2013. **1**(10): p. 837-845.
63. Gertz, M.A., et al., *Diagnosis, Prognosis, and Therapy of Transthyretin Amyloidosis.* Journal of the American College of Cardiology, 2015. **66**(21): p. 2451-2466.
64. Adams, D., *Review of the recent literature on peripheral neuropathies: Therapeutic advances.* Revue Neurologique, 2013. **169**(12): p. 1004-1009.
65. Lobato, L., et al., *Familial amyloid polyneuropathy type I (Portuguese): distribution and characterization of renal amyloid deposits.* Am J Kidney Dis, 1998. **31**(6): p. 940-6.
66. Sousa, A., et al., *Familial amyloidotic polyneuropathy in Sweden: geographical distribution, age of onset, and prevalence.* Hum Hered, 1993. **43**(5): p. 288-94.
67. Pinney, J.H. and P.N. Hawkins, *Amyloidosis.* Ann Clin Biochem, 2012. **49**(Pt 3): p. 229-41.

68. Ando, Y., et al., *Guideline of transthyretin-related hereditary amyloidosis for clinicians.* Orphanet J Rare Dis, 2013. **8**: p. 31.
69. Rapezzi, C., et al., *Transthyretin-related amyloidoses and the heart: a clinical overview.* Nat Rev Cardiol, 2010. **7**(7): p. 398-408.
70. Staunton, H., et al., *Hereditary amyloid polyneuropathy in north west Ireland.* Brain, 1987. **110 (Pt 5)**: p. 1231-45.
71. Jacobson, D.R., et al., *Prevalence of the amyloidogenic transthyretin (TTR) V122I allele in 14 333 African-Americans.* Amyloid, 2015. **22**(3): p. 171-4.
72. Jacobson, D.R., et al., *Revised transthyretin Ile 122 allele frequency in African-Americans.* Hum Genet, 1996. **98**(2): p. 236-8.
73. Buxbaum, J., et al., *Transthyretin V122I in African Americans with congestive heart failure.* J Am Coll Cardiol, 2006. **47**(8): p. 1724-5.
74. Holmgren, G., et al., *Biochemical effect of liver transplantation in two Swedish patients with familial amyloidotic polyneuropathy (FAP-met30).* Clin Genet, 1991. **40**(3): p. 242-6.
75. Wilczek, H.E., et al., *Long-term data from the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR).* Amyloid, 2011. **18 Suppl 1**: p. 193-5.
76. Herlenius, G., et al., *Ten years of international experience with liver transplantation for familial amyloidotic polyneuropathy: results from the Familial Amyloidotic Polyneuropathy World Transplant Registry.* Transplantation, 2004. **77**(1): p. 64-71.
77. Benson, M.D., *Liver transplantation and transthyretin amyloidosis.* Muscle Nerve, 2013. **47**(2): p. 157-62.
78. Liepnieks, J.J. and M.D. Benson, *Progression of cardiac amyloid deposition in hereditary transthyretin amyloidosis patients after liver transplantation.* Amyloid, 2007. **14**(4): p. 277-82.
79. Drent, G., et al., *Quality of life in patients with familial amyloidotic polyneuropathy long-term after liver transplantation.* Amyloid, 2009. **16**(3): p. 133-41.
80. Waddington Cruz, M. and M.D. Benson, *A Review of Tafamidis for the Treatment of Transthyretin-Related Amyloidosis.* Neurol Ther, 2015. **4**(2): p. 61-79.
81. Sayed, R.H., P.N. Hawkins, and H.J. Lachmann, *Emerging treatments for amyloidosis.* Kidney Int, 2015. **87**(3): p. 516-26.
82. Sattianayagam, P., et al., *Systemic AL (light-chain) amyloidosis and the gastrointestinal tract.* Scand J Gastroenterol, 2009. **44**(11): p. 1384-5.
83. Kwiatkowski, D.J., et al., *Localization of gelsolin proximal to ABL on chromosome 9.* Am J Hum Genet, 1988. **42**(4): p. 565-72.
84. Kiuru-Enari, S. and M. Haltia, *Hereditary gelsolin amyloidosis.* Handb Clin Neurol, 2013. **115**: p. 659-81.
85. Yin, H.L. and T.P. Stossel, *Purification and structural properties of gelsolin, a Ca2+-activated regulatory protein of macrophages.* J Biol Chem, 1980. **255**(19): p. 9490-3.
86. Pihlamaa, T., et al., *Gelsolin amyloidosis as a cause of early aging and progressive bilateral facial paralysis.* Plast Reconstr Surg, 2011. **127**(6): p. 2342-51.
87. Kiuru, S., O. Salonen, and M. Haltia, *Gelsolin-related spinal and cerebral amyloid angiopathy.* Ann Neurol, 1999. **45**(3): p. 305-11.
88. Koskelainen, S., et al., *Gelsolin amyloid angiopathy causes severe disruption of the arterial wall.* APMIS, 2016. **124**(8): p. 639-48.
89. Ardalan, M.R., M.M. Shoja, and S. Kiuru-Enari, *Amyloidosis-related nephrotic syndrome due to a G654A gelsolin mutation: the first report from the Middle East.* Nephrology Dialysis Transplantation, 2007. **22**(1): p. 272-275.

90. Kiuru-Enari, S. and M. Haltia, *Hereditary gelsolin amyloidosis.* Peripheral Nerve Disorders, 2013. **115**: p. 659-681.
91. Kiuru, S., *Gelsolin-related familial amyloidosis, Finnish type (FAF), and its variants found worldwide.* Amyloid-Journal of Protein Folding Disorders, 1998. **5**(1): p. 55-66.
92. Schmidt, E.K., et al., *Causes of death and life span in Finnish gelsolin amyloidosis.* Annals of Medicine, 2016. **48**(5): p. 352-358.
93. Shoja, M.M., et al., *Outcome of renal transplant in hereditary gelsolin amyloidosis.* Am J Med Sci, 2009. **337**(5): p. 370-2.
94. Gillmore, J.D., et al., *Diagnosis, Pathogenesis, Treatment, and Prognosis of Hereditary Fibrinogen A alpha-Chain Amyloidosis.* Journal of the American Society of Nephrology, 2009. **20**(2): p. 444-451.
95. Benson, M.D., et al., *Hereditary Renal Amyloidosis Associated with a Mutant Fibrinogen Alpha-Chain.* Nature Genetics, 1993. **3**(3): p. 252-255.
96. Picken, M.M. and R.P. Linke, *Nephrotic syndrome due to an amyloidogenic mutation in fibrinogen A alpha chain.* J Am Soc Nephrol, 2009. **20**(8): p. 1681-5.
97. Larsen, C.P., et al., *Prevalence and morphology of leukocyte chemotactic factor 2-associated amyloid in renal biopsies.* Kidney Int, 2010. **77**(9): p. 816-9.
98. Said, S.M., et al., *Renal Amyloidosis: Origin and Clinicopathologic Correlations of 474 Recent Cases.* Clinical Journal of the American Society of Nephrology, 2013. **8**(9): p. 1515-1523.
99. Larsen, C.P., et al., *Clinical, morphologic, and genetic features of renal leukocyte chemotactic factor 2 amyloidosis.* Kidney Int, 2014. **86**(2): p. 378-82.
100. Larsen, C.P., et al., *Prevalence and organ distribution of leukocyte chemotactic factor 2 amyloidosis (ALECT2) among decedents in New Mexico.* Amyloid-Journal of Protein Folding Disorders, 2016. **23**(2): p. 119-123.
101. Murphy, C.L., et al., *Leukocyte chemotactic factor 2 (LECT2)-associated renal amyloidosis: a case series.* Am J Kidney Dis, 2010. **56**(6): p. 1100-7.
102. Said, S.M., et al., *Characterization and outcomes of renal leukocyte chemotactic factor 2-associated amyloidosis.* Kidney Int, 2014. **86**(2): p. 370-7.
103. Benson, M.D., *Amyloidosis*, in *In The Metabolic and Molecular Bases of Inherited Disease*, C.R. Scriver, et al., Editors. 1995, McGraw Hill: New York. p. 1459-91.
104. Mahmood, S., et al., *Natural history and outcomes in localised immunoglobulin light-chain amyloidosis: a long-term observational study.* Lancet Haematol, 2015. **2**(6): p. e241-50.
105. Mohty, D., et al., *Cardiac amyloidosis: updates in diagnosis and management.* Arch Cardiovasc Dis, 2013. **106**(10): p. 528-40.
106. Gertz, M.A., et al., *Definition of organ involvement and treatment response in immunoglobulin light chain amyloidosis (AL): a consensus opinion from the 10th International Symposium on Amyloid and Amyloidosis, Tours, France, 18-22 April 2004.* Am J Hematol, 2005. **79**(4): p. 319-28.
107. Poels, M.M., et al., *Incidence of cerebral microbleeds in the general population: the Rotterdam Scan Study.* Stroke, 2011. **42**(3): p. 656-61.
108. Adams, D., *Hereditary and acquired amyloid neuropathies.* J Neurol, 2001. **248**(8): p. 647-57.
109. Kyle, R.A. and M.A. Gertz, *Primary systemic amyloidosis: clinical and laboratory features in 474 cases.* Semin Hematol, 1995. **32**(1): p. 45-59.
110. Benson, M.D. and J.C. Kincaid, *The molecular biology and clinical features of amyloid neuropathy.* Muscle Nerve, 2007. **36**(4): p. 411-23.

111. Carrwik, C. and U. Stenevi, *Lattice corneal dystrophy, gelsolin type (Meretoja's syndrome).* Acta Ophthalmol, 2009. **87**(8): p. 813-9.
112. Joy, T., et al., *APOA1 related amyloidosis: a case report and literature review.* Clin Biochem, 2003. **36**(8): p. 641-5.
113. Weiss, B.M., et al., *Increased serum free light chains precede the presentation of immunoglobulin light chain amyloidosis.* J Clin Oncol, 2014. **32**(25): p. 2699-704.
114. Abraham, R.S., et al., *Quantitative analysis of serum free light chains. A new marker for the diagnostic evaluation of primary systemic amyloidosis.* Am J Clin Pathol, 2003. **119**(2): p. 274-8.
115. Katzmann, J.A., et al., *Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains.* Clin Chem, 2002. **48**(9): p. 1437-44.
116. Palladini, G., et al., *A staging system for renal outcome and early markers of renal response to chemotherapy in AL amyloidosis.* Blood, 2014. **124**(15): p. 2325-32.
117. Palladini, G., et al., *New criteria for response to treatment in immunoglobulin light chain amyloidosis based on free light chain measurement and cardiac biomarkers: impact on survival outcomes.* J Clin Oncol, 2012. **30**(36): p. 4541-9.
118. Gillmore, J.D., et al., *Nonbiopsy Diagnosis of Cardiac Transthyretin Amyloidosis.* Circulation, 2016. **133**(24): p. 2404-12.
119. Kumar, S., et al., *Revised prognostic staging system for light chain amyloidosis incorporating cardiac biomarkers and serum free light chain measurements.* J Clin Oncol, 2012. **30**(9): p. 989-95.
120. Dispenzieri, A., et al., *Survival in patients with primary systemic amyloidosis and raised serum cardiac troponins.* Lancet, 2003. **361**(9371): p. 1787-9.
121. Hunt, P.J., et al., *The Amino-Terminal Portion of Pro-Brain Natriuretic Peptide (Pro-Bnp) Circulates in Human Plasma.* Biochemical and Biophysical Research Communications, 1995. **214**(3): p. 1175-1183.
122. Palladini, G., et al., *Serum N-terminal pro-brain natriuretic peptide is a sensitive marker of myocardial dysfunction in AL amyloidosis.* Circulation, 2003. **107**(19): p. 2440-2445.
123. Wechalekar, A.D., et al., *A European collaborative study of treatment outcomes in 346 patients with cardiac stage III AL amyloidosis.* Blood, 2013. **121**(17): p. 3420-3427.
124. Palladini, G., et al., *Treatment with oral melphalan plus dexamethasone produces long-term remissions in AL amyloidosis.* Blood, 2007. **110**(2): p. 787-788.
125. Banypersad, S.M., et al., *Updates in Cardiac Amyloidosis: A Review.* Journal of the American Heart Association, 2012. **1**(2).
126. Rahman, J.E., et al., *Noninvasive diagnosis of biopsy-proven cardiac amyloidosis.* Journal of the American College of Cardiology, 2004. **43**(3): p. 410-415.
127. Bejar, D., et al., *Infiltrative Cardiomyopathies.* Clin Med Insights Cardiol, 2015. **9**(Suppl 2): p. 29-38.
128. Huang, J., et al., *Contribution of Electrocardiogram in the Differentiation of Cardiac Amyloidosis and Nonobstructive Hypertrophic Cardiomyopathy.* Int Heart J, 2015. **56**(5): p. 522-6.
129. Palladini, G., et al., *Holter monitoring in AL amyloidosis: prognostic implications.* Pacing Clin Electrophysiol, 2001. **24**(8 Pt 1): p. 1228-33.
130. Zou, Z., et al., *Nephrogenic systemic fibrosis: review of 370 biopsy-confirmed cases.* JACC Cardiovasc Imaging, 2011. **4**(11): p. 1206-16.
131. Maceira, A.M., et al., *Cardiovascular magnetic resonance in cardiac amyloidosis.* Circulation, 2005. **111**(2): p. 186-93.

132. Banypersad, S.M., et al., *Quantification of myocardial extracellular volume fraction in systemic AL amyloidosis: an equilibrium contrast cardiovascular magnetic resonance study.* Circ Cardiovasc Imaging, 2013. **6**(1): p. 34-9.
133. Fontana, M., et al., *Prognostic Value of Late Gadolinium Enhancement Cardiovascular Magnetic Resonance in Cardiac Amyloidosis.* Circulation, 2015. **132**(16): p. 1570-9.
134. Fontana, M., et al., *Cardiovascular magnetic resonance for amyloidosis.* Heart Fail Rev, 2015. **20**(2): p. 133-44.
135. Ferreira, P.F., et al., *Cardiovascular magnetic resonance artefacts.* J Cardiovasc Magn Reson, 2013. **15**: p. 41.
136. Tsang, W. and R.M. Lang, *Echocardiographic evaluation of cardiac amyloid.* Curr Cardiol Rep, 2010. **12**(3): p. 272-6.
137. Park, S.J., et al., *Left ventricular torsion by two-dimensional speckle tracking echocardiography in patients with diastolic dysfunction and normal ejection fraction.* J Am Soc Echocardiogr, 2008. **21**(10): p. 1129-37.
138. Feng, D., et al., *Intracardiac thrombosis and embolism in patients with cardiac amyloidosis.* Circulation, 2007. **116**(21): p. 2420-6.
139. Zubkov, A.Y., et al., *Primary systemic amyloidosis with ischemic stroke as a presenting complication.* Neurology, 2007. **69**(11): p. 1136-1141.
140. Leung, N., et al., *A detailed evaluation of the current renal response criteria in AL amyloidosis: is it time for a revision?* Haematologica, 2013. **98**(6): p. 988-92.
141. Hawkins, P.N., et al., *Diagnostic radionuclide imaging of amyloid: biological targeting by circulating human serum amyloid P component.* Lancet, 1988. **1**(8600): p. 1413-8.
142. Hazenberg, B.P., et al., *Diagnostic performance of 123I-labeled serum amyloid P component scintigraphy in patients with amyloidosis.* Am J Med, 2006. **119**(4): p. 355 e15-24.
143. Sachchithanantham, S. and A.D. Wechalekar, *Imaging in systemic amyloidosis.* Br Med Bull, 2013. **107**: p. 41-56.
144. Hawkins, P.N., J.P. Lavender, and M.B. Pepys, *Evaluation of systemic amyloidosis by scintigraphy with 123I-labeled serum amyloid P component.* N Engl J Med, 1990. **323**(8): p. 508-13.
145. Hawkins, P.N., R. Wootton, and M.B. Pepys, *Metabolic studies of radioiodinated serum amyloid P component in normal subjects and patients with systemic amyloidosis.* J Clin Invest, 1990. **86**(6): p. 1862-9.
146. Lovat, L.B., et al., *The liver in systemic amyloidosis: insights from 123I serum amyloid P component scintigraphy in 484 patients.* Gut, 1998. **42**(5): p. 727-34.
147. Yao, Y., et al., *A clinicopathological analysis in a large cohort of Chinese patients with renal amyloid light-chain amyloidosis.* Nephrol Dial Transplant, 2013. **28**(3): p. 689-97.
148. Perugini, E., et al., *Noninvasive etiologic diagnosis of cardiac amyloidosis using 99mTc-3,3-diphosphono-1,2-propanodicarboxylic acid scintigraphy.* J Am Coll Cardiol, 2005. **46**(6): p. 1076-84.
149. Rapezzi, C., et al., *Usefulness and limitations of 99mTc-3,3-diphosphono-1,2-propanodicarboxylic acid scintigraphy in the aetiological diagnosis of amyloidotic cardiomyopathy.* Eur J Nucl Med Mol Imaging, 2011. **38**(3): p. 470-8.
150. Gertz, M.A., et al., *Autologous stem cell transplant for immunoglobulin light chain amyloidosis: a status report.* Leuk Lymphoma, 2010. **51**(12): p. 2181-7.
151. Lachmann, H.J., et al., *Misdiagnosis of hereditary amyloidosis as AL (primary) amyloidosis.* N Engl J Med, 2002. **346**(23): p. 1786-91.

152. Gilbertson, J.A., et al., *A comparison of immunohistochemistry and mass spectrometry for determining the amyloid fibril protein from formalin-fixed biopsy tissue.* J Clin Pathol, 2015. **68**(4): p. 314-7.
153. Lavatelli, F. and J.A. Vrana, *Proteomic typing of amyloid deposits in systemic amyloidoses.* Amyloid, 2011. **18**(4): p. 177-82.
154. Sethi, S., et al., *Laser microdissection and mass spectrometry-based proteomics aids the diagnosis and typing of renal amyloidosis.* Kidney Int, 2012. **82**(2): p. 226-34.
155. Loo, D., et al., *Proteomics in molecular diagnosis: typing of amyloidosis.* J Biomed Biotechnol, 2011. **2011**: p. 754109.
156. Mann, M. and M. Wilm, *Error-tolerant identification of peptides in sequence databases by peptide sequence tags.* Anal Chem, 1994. **66**(24): p. 4390-9.
157. Addona, T. and K. Clauser, *De novo peptide sequencing via manual interpretation of MS/MS spectra.* Curr Protoc Protein Sci, 2002. **Chapter 16**: p. Unit 16 11.
158. Hawkins, P.N., *Studies with radiolabelled serum amyloid P component provide evidence for turnover and regression of amyloid deposits in vivo.* Clin Sci (Lond), 1994. **87**(3): p. 289-95.
159. Rubinow, A., M. Skinner, and A.S. Cohen, *Digoxin sensitivity in amyloid cardiomyopathy.* Circulation, 1981. **63**(6): p. 1285-8.
160. Gertz, M.A., et al., *Worsening of congestive heart failure in amyloid heart disease treated by calcium channel-blocking agents.* Am J Cardiol, 1985. **55**(13 Pt 1): p. 1645.
161. Pollak, A. and R.H. Falk, *Left ventricular systolic dysfunction precipitated by verapamil in cardiac amyloidosis.* Chest, 1993. **104**(2): p. 618-20.
162. Falk, R.H., *Diagnosis and management of the cardiac amyloidoses.* Circulation, 2005. **112**(13): p. 2047-60.
163. Jaccard, A., et al., *High-dose melphalan versus melphalan plus dexamethasone for AL amyloidosis.* N Engl J Med, 2007. **357**(11): p. 1083-93.
164. Dispenzieri, A., et al., *Poor tolerance to high doses of thalidomide in patients with primary systemic amyloidosis.* Amyloid, 2003. **10**(4): p. 257-61.
165. Wechalekar, A.D., et al., *Safety and efficacy of risk-adapted cyclophosphamide, thalidomide, and dexamethasone in systemic AL amyloidosis.* Blood, 2007. **109**(2): p. 457-64.
166. Palladini, G., et al., *Salvage therapy with lenalidomide and dexamethasone in patients with advanced AL amyloidosis refractory to melphalan, bortezomib, and thalidomide.* Ann Hematol, 2012. **91**(1): p. 89-92.
167. Baz, R., et al., *Single agent lenalidomide in newly diagnosed multiple myeloma: a retrospective analysis.* Leuk Lymphoma, 2010. **51**(6): p. 1015-9.
168. Specter, R., et al., *Kidney dysfunction during lenalidomide treatment for AL amyloidosis.* Nephrol Dial Transplant, 2011. **26**(3): p. 881-6.
169. Dispenzieri, A., et al., *Discordance between serum cardiac biomarker and immunoglobulin-free light-chain response in patients with immunoglobulin light-chain amyloidosis treated with immune modulatory drugs.* Am J Hematol, 2010. **85**(10): p. 757-9.
170. Gatt, M.E. and G. Palladini, *Light chain amyloidosis 2012: a new era.* Br J Haematol, 2013. **160**(5): p. 582-98.
171. Dispenzieri, A., et al., *Activity of pomalidomide in patients with immunoglobulin light-chain amyloidosis.* Blood, 2012. **119**(23): p. 5397-404.
172. Bianchi, G. and I.M. Ghobrial, *Molecular mechanisms of effectiveness of novel therapies in multiple myeloma.* Leuk Lymphoma, 2013. **54**(2): p. 229-41.
173. Mahmood, S., et al., *Update on treatment of light chain amyloidosis.* Haematologica, 2014. **99**(2): p. 209-21.

174. Venner, C.P., et al., *Cyclophosphamide, bortezomib, and dexamethasone therapy in AL amyloidosis is associated with high clonal response rates and prolonged progression-free survival.* Blood, 2012. **119**(19): p. 4387-90.
175. Kastritis, E., et al., *Bortezomib with or without dexamethasone in primary systemic (light chain) amyloidosis.* J Clin Oncol, 2010. **28**(6): p. 1031-7.
176. Palladini, G., et al., *A European collaborative study of cyclophosphamide, bortezomib, and dexamethasone in upfront treatment of systemic AL amyloidosis.* Blood, 2015. **126**(5): p. 612-5.
177. Reece, D.E., et al., *Efficacy and safety of once-weekly and twice-weekly bortezomib in patients with relapsed systemic AL amyloidosis: results of a phase 1/2 study.* Blood, 2011. **118**(4): p. 865-73.
178. Sanchorawala, V., et al., *A phase 1/2 study of the oral proteasome inhibitor ixazomib in relapsed or refractory light-chain (AL) amyloidosis.* Blood, 2017.
179. Tennent, G.A., L.B. Lovat, and M.B. Pepys, *Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis.* Proc Natl Acad Sci U S A, 1995. **92**(10): p. 4299-303.
180. Mollee, P., et al., *A phase II study of risk-adapted intravenous melphalan in patients with AL amyloidosis.* Br J Haematol, 2012. **157**(6): p. 766-9.
181. Gillmore, J.D., et al., *Sustained pharmacological depletion of serum amyloid P component in patients with systemic amyloidosis.* Br J Haematol, 2010. **148**(5): p. 760-7.
182. Dispenzieri, A., et al., *Serum cardiac troponins and N-terminal pro-brain natriuretic peptide: a staging system for primary systemic amyloidosis.* J Clin Oncol, 2004. **22**(18): p. 3751-7.
183. Dubrey, S.W., et al., *Familial and primary (AL) cardiac amyloidosis: echocardiographically similar diseases with distinctly different clinical outcomes.* Heart, 1997. **78**(1): p. 74-82.
184. Lin, G., A. Dispenzieri, and P.A. Brady, *Successful termination of a ventricular arrhythmia by implantable cardioverter defibrillator therapy in a patient with cardiac amyloidosis: insight into mechanisms of sudden death.* Eur Heart J, 2010. **31**(12): p. 1538.
185. Kristen, A.V., et al., *Prophylactic implantation of cardioverter-defibrillator in patients with severe cardiac amyloidosis and high risk for sudden cardiac death.* Heart Rhythm, 2008. **5**(2): p. 235-40.
186. Falk, R.H., A. Rubinow, and A.S. Cohen, *Cardiac arrhythmias in systemic amyloidosis: correlation with echocardiographic abnormalities.* J Am Coll Cardiol, 1984. **3**(1): p. 107-13.
187. Varr, B.C., et al., *Implantable cardioverter-defibrillator placement in patients with cardiac amyloidosis.* Heart Rhythm, 2014. **11**(1): p. 158-62.
188. Lin, G., et al., *Implantable cardioverter defibrillators in patients with cardiac amyloidosis.* J Cardiovasc Electrophysiol, 2013. **24**(7): p. 793-8.
189. Dhoble, A., et al., *Cardiac amyloidosis treated with an implantable cardioverter defibrillator and subcutaneous array lead system: report of a case and literature review.* Clin Cardiol, 2009. **32**(8): p. E63-5.
190. Zaks, N., et al., *Analysis of the three most common MEFV mutations in 412 patients with familial Mediterranean fever.* Isr Med Assoc J, 2003. **5**(8): p. 585-8.
191. Tunca, M., et al., *Familial Mediterranean fever (FMF) in Turkey: results of a nationwide multicenter study.* Medicine (Baltimore), 2005. **84**(1): p. 1-11.
192. *The registry of Hereditary Auto-inflammatory Disorders Mutations*. 2017, International Society for Systemic AutoInflammatory Diseases.
193. Ozen, S. and Y. Bilginer, *A clinical guide to autoinflammatory diseases: familial Mediterranean fever and next-of-kin.* Nat Rev Rheumatol, 2014. **10**(3): p. 135-47.

194. Stojanov, S. and D.L. Kastner, *Familial autoinflammatory diseases: genetics, pathogenesis and treatment.* Curr Opin Rheumatol, 2005. **17**(5): p. 586-99.
195. Goldfinger, S.E., *Colchicine for familial Mediterranean fever.* N Engl J Med, 1972. **287**(25): p. 1302.
196. Livneh, A., et al., *Colchicine treatment of AA amyloidosis of familial Mediterranean fever. An analysis of factors affecting outcome.* Arthritis Rheum, 1994. **37**(12): p. 1804-11.
197. Lidar, M., et al., *Colchicine nonresponsiveness in familial Mediterranean fever: clinical, genetic, pharmacokinetic, and socioeconomic characterization.* Semin Arthritis Rheum, 2004. **33**(4): p. 273-82.
198. Gafni, J., M. Ravid, and E. Sohar, *The role of amyloidosis in familial mediterranean fever. A population study.* Isr J Med Sci, 1968. **4**(5): p. 995-9.
199. Sackett, D.L. and J.K. Varma, *Molecular mechanism of colchicine action: induced local unfolding of beta-tubulin.* Biochemistry, 1993. **32**(49): p. 13560-5.
200. Vandecandelaere, A., S.R. Martin, and Y. Engelborghs, *Response of microtubules to the addition of colchicine and tubulin-colchicine: evaluation of models for the interaction of drugs with microtubules.* Biochem J, 1997. **323 (Pt 1)**: p. 189-96.
201. Paya, M., et al., *Involvement of secretory phospholipase A2 activity in the zymosan rat air pouch model of inflammation.* Br J Pharmacol, 1996. **117**(8): p. 1773-9.
202. Zurier, R.B., S. Hoffstein, and G. Weissmann, *Mechanisms of lysosomal enzyme release from human leukocytes. I. Effect of cyclic nucleotides and colchicine.* J Cell Biol, 1973. **58**(1): p. 27-41.
203. Wright, D.G. and S.E. Malawista, *Mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis: inhibition by colchicine and cortisol but not by salicylate.* Arthritis Rheum, 1973. **16**(6): p. 749-58.
204. Ben-Chetrit, E. and S. Aamar, *About colchicine compliance, resistance and virulence.* Clin Exp Rheumatol, 2009. **27**(2 Suppl 53): p. S1-3.
205. Polat, A., et al., *Comparison of the efficacy of once- and twice-daily colchicine dosage in pediatric patients with familial Mediterranean fever--a randomized controlled noninferiority trial.* Arthritis Res Ther, 2016. **18**: p. 85.
206. Amital, H. and E. Ben-Chetrit, *Therapeutic approaches to familial Mediterranean fever. What do we know and where are we going to?* Clin Exp Rheumatol, 2004. **22**(4 Suppl 34): p. S4-7.
207. Kallinich, T., et al., *Colchicine use in children and adolescents with familial Mediterranean fever: literature review and consensus statement.* Pediatrics, 2007. **119**(2): p. e474-83.
208. Roldan, R., et al., *Anakinra: new therapeutic approach in children with Familial Mediterranean Fever resistant to colchicine.* Joint Bone Spine, 2008. **75**(4): p. 504-5.
209. Lidar, M., et al., *Intravenous colchicine for treatment of patients with familial Mediterranean fever unresponsive to oral colchicine.* J Rheumatol, 2003. **30**(12): p. 2620-3.
210. Dember, L.M., et al., *Eprodisate for the treatment of renal disease in AA amyloidosis.* N Engl J Med, 2007. **356**(23): p. 2349-60.
211. Rumjon, A., T. Coats, and M.M. Javaid, *Review of eprodisate for the treatment of renal disease in AA amyloidosis.* Int J Nephrol Renovasc Dis, 2012. **5**: p. 37-43.
212. Bulua, A.C., et al., *Efficacy of etanercept in the tumor necrosis factor receptor-associated periodic syndrome: a prospective, open-label, dose-escalation study.* Arthritis Rheum, 2012. **64**(3): p. 908-13.
213. Nakamura, T., et al., *Effectiveness of etanercept vs cyclophosphamide as treatment for patients with amyloid A amyloidosis secondary to rheumatoid arthritis.* Rheumatology (Oxford), 2012. **51**(11): p. 2064-9.

214. Aganna, E., et al., *Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis.* Arthritis Rheum, 2002. **46**(9): p. 2445-52.
215. Stankovic Stojanovic, K., et al., *Dramatic beneficial effect of interleukin-1 inhibitor treatment in patients with familial Mediterranean fever complicated with amyloidosis and renal failure.* Nephrol Dial Transplant, 2012. **27**(5): p. 1898-901.
216. Mitroulis, I., et al., *Anakinra suppresses familial Mediterranean fever crises in a colchicine-resistant patient.* Neth J Med, 2008. **66**(11): p. 489-91.
217. Moser, C., et al., *Successful treatment of familial Mediterranean fever with Anakinra and outcome after renal transplantation.* Nephrol Dial Transplant, 2009. **24**(2): p. 676-8.
218. Hashkes, P.J., et al., *Rilonacept for colchicine-resistant or -intolerant familial Mediterranean fever: a randomized trial.* Ann Intern Med, 2012. **157**(8): p. 533-41.
219. Hoffman, H.M., *Rilonacept for the treatment of cryopyrin-associated periodic syndromes (CAPS).* Expert Opin Biol Ther, 2009. **9**(4): p. 519-31.
220. Alten, R., et al., *Efficacy and safety of the human anti-IL-1beta monoclonal antibody canakinumab in rheumatoid arthritis: results of a 12-week, Phase II, dose-finding study.* BMC Musculoskelet Disord, 2011. **12**: p. 153.
221. Scarpioni, R., et al., *Renal involvement in secondary amyloidosis of Muckle-Wells syndrome: marked improvement of renal function and reduction of proteinuria after therapy with human anti-interleukin-1beta monoclonal antibody canakinumab.* Clin Rheumatol, 2015. **34**(7): p. 1311-6.
222. Lachmann, H.J., et al., *Use of canakinumab in the cryopyrin-associated periodic syndrome.* N Engl J Med, 2009. **360**(23): p. 2416-25.
223. Schlesinger, N., et al., *Canakinumab for acute gouty arthritis in patients with limited treatment options: results from two randomised, multicentre, active-controlled, double-blind trials and their initial extensions.* Ann Rheum Dis, 2012. **71**(11): p. 1839-48.
224. Kuemmerle-Deschner, J.B., et al., *Canakinumab (ACZ885, a fully human IgG1 anti-IL-1beta mAb) induces sustained remission in pediatric patients with cryopyrin-associated periodic syndrome (CAPS).* Arthritis Res Ther, 2011. **13**(1): p. R34.
225. Miyagawa, I., et al., *Study on the safety and efficacy of tocilizumab, an anti-IL-6 receptor antibody, in patients with rheumatoid arthritis complicated with AA amyloidosis.* Mod Rheumatol, 2014. **24**(3): p. 405-9.
226. Kisilevsky, R., *The relation of proteoglycans, serum amyloid P and apo E to amyloidosis current status, 2000.* Amyloid, 2000. **7**(1): p. 23-5.
227. Miller, S.R., Y. Sekijima, and J.W. Kelly, *Native state stabilization by NSAIDs inhibits transthyretin amyloidogenesis from the most common familial disease variants.* Lab Invest, 2004. **84**(5): p. 545-52.
228. Sekijima, Y., M.A. Dendle, and J.W. Kelly, *Orally administered diflunisal stabilizes transthyretin against dissociation required for amyloidogenesis.* Amyloid, 2006. **13**(4): p. 236-49.
229. Berk, J.L., et al., *Repurposing diflunisal for familial amyloid polyneuropathy: a randomized clinical trial.* JAMA, 2013. **310**(24): p. 2658-67.
230. Bulawa, C.E., et al., *Tafamidis, a potent and selective transthyretin kinetic stabilizer that inhibits the amyloid cascade.* Proc Natl Acad Sci U S A, 2012. **109**(24): p. 9629-34.
231. Coelho, T., et al., *Tafamidis for transthyretin familial amyloid polyneuropathy: a randomized, controlled trial.* Neurology, 2012. **79**(8): p. 785-92.
232. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.

233. Goodchild, J., *Therapeutic oligonucleotides.* Methods Mol Biol, 2011. **764**: p. 1-15.
234. Sehgal, A., A. Vaishnaw, and K. Fitzgerald, *Liver as a target for oligonucleotide therapeutics.* J Hepatol, 2013. **59**(6): p. 1354-9.
235. Akinc, A., et al., *Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms.* Mol Ther, 2010. **18**(7): p. 1357-64.
236. Loke, S.L., et al., *Characterization of oligonucleotide transport into living cells.* Proc Natl Acad Sci U S A, 1989. **86**(10): p. 3474-8.
237. Elbashir, S.M., W. Lendeckel, and T. Tuschl, *RNA interference is mediated by 21- and 22-nucleotide RNAs.* Genes Dev, 2001. **15**(2): p. 188-200.
238. Yazaki, M., et al., *Cardiac amyloid in patients with familial amyloid polyneuropathy consists of abundant wild-type transthyretin.* Biochem Biophys Res Commun, 2000. **274**(3): p. 702-6.
239. Coelho, T., et al., *Long-term effects of tafamidis for the treatment of transthyretin familial amyloid polyneuropathy.* J Neurol, 2013. **260**(11): p. 2802-14.
240. Raal, F.J., et al., *Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial.* Lancet, 2010. **375**(9719): p. 998-1006.
241. Chia, S., et al., *Phase II Trial of OGX-011 in Combination with Docetaxel in Metastatic Breast Cancer.* Clinical Cancer Research, 2009. **15**(2): p. 708-713.
242. Hair, P., F. Cameron, and K. McKeage, *Mipomersen Sodium: First Global Approval.* Drugs, 2013. **73**(5): p. 487-493.
243. Ackermann, E.J., et al., *Clinical development of an antisense therapy for the treatment of transthyretin-associated polyneuropathy.* Amyloid-Journal of Protein Folding Disorders, 2012. **19**: p. 43-44.
244. Kluve-Beckerman, B. and M.D. Benson, *AA amyloidosis: potential therapy with antisense oligonucleotides.* Amyloid-Journal of Protein Folding Disorders, 2010. **17**: p. 117-117.
245. Pinney, J.H., et al., *Renal transplantation in systemic amyloidosis-importance of amyloid fibril type and precursor protein abundance.* Am J Transplant, 2013. **13**(2): p. 433-41.
246. Sahutoglu, T., et al., *Comparative Analysis of Outcomes of Kidney Transplantation in Patients With AA Amyloidosis and Chronic Glomerulonephritis.* Transplant Proc, 2016. **48**(6): p. 2011-6.
247. Kofman, T., et al., *Renal transplantation in patients with AA amyloidosis nephropathy: results from a French multicenter study.* Am J Transplant, 2011. **11**(11): p. 2423-31.
248. Haq, A., et al., *Complications of renal transplantation in patients with amyloidosis.* Transplant Proc, 2007. **39**(1): p. 120-4.
249. Traynor, C.A., et al., *Clinical and pathologic characteristics of hereditary apolipoprotein A-I amyloidosis in Ireland.* Nephrology (Carlton), 2013. **18**(8): p. 549-54.
250. Gillmore, J.D., et al., *Organ transplantation in hereditary apolipoprotein AI amyloidosis.* Am J Transplant, 2006. **6**(10): p. 2342-7.
251. Gillmore, J.D., et al., *Hereditary fibrinogen A alpha-chain amyloidosis: clinical phenotype and role of liver transplantation.* Blood, 2010. **115**(21): p. 4313; author reply 4314-5.
252. Yamashita, T., et al., *Long-term survival after liver transplantation in patients with familial amyloid polyneuropathy.* Neurology, 2012. **78**(9): p. 637-43.
253. Solomon, A., C.L. Murphy, and P. Westermark, *Unreliability of immunohistochemistry for typing amyloid deposits.* Arch Pathol Lab Med, 2008. **132**(1): p. 14; author reply 14-5.
254. Wu, I. and C.R. Parikh, *Screening for kidney diseases: older measures versus novel biomarkers.* Clin J Am Soc Nephrol, 2008. **3**(6): p. 1895-901.

255. Li, F., et al., *Urinary retinol binding protein is a potential biomarker for renal function in primary systemic amyloidosis: A retrospective study.* Leuk Lymphoma, 2015: p. 1-3.
256. Wechalekar, A.D., J.D. Gillmore, and P.N. Hawkins, *Systemic amyloidosis.* Lancet, 2016. **387**(10038): p. 2641-54.
257. Kusumoto, F.M., et al., *HRS/ACC/AHA expert consensus statement on the use of implantable cardioverter-defibrillator therapy in patients who are not included or not well represented in clinical trials.* J Am Coll Cardiol, 2014. **64**(11): p. 1143-77.
258. Randall, R.E., et al., *Manifestations of Systemic Light Chain Deposition.* American Journal of Medicine, 1976. **60**(2): p. 293-299.
259. Trikas, A., et al., *Comparison of usefulness between exercise capacity and echocardiographic indexes of left ventricular function in cardiac amyloidosis.* American Journal of Cardiology, 1999. **84**(9): p. 1049-1054.
260. Crapo, R.O., et al., *ATS statement: Guidelines for the six-minute walk test.* American Journal of Respiratory and Critical Care Medicine, 2002. **166**(1): p. 111-117.
261. Oken, M.M., et al., *Toxicity and Response Criteria of the Eastern-Cooperative-Oncology-Group.* American Journal of Clinical Oncology-Cancer Clinical Trials, 1982. **5**(6): p. 649-655.
262. Banypersad, S.M., et al., *T1 mapping and survival in systemic light-chain amyloidosis.* Eur Heart J, 2015. **36**(4): p. 244-51.
263. Koyama, J., P.A. Ray-Sequin, and R.H. Falk, *Longitudinal myocardial function assessed by tissue velocity, strain, and strain rate tissue Doppler echocardiography in patients with AL (primary) cardiac amyloidosis.* Circulation, 2003. **107**(19): p. 2446-2452.
264. Buss, S.J., et al., *Longitudinal left ventricular function for prediction of survival in systemic light-chain amyloidosis: incremental value compared with clinical and biochemical markers.* J Am Coll Cardiol, 2012. **60**(12): p. 1067-76.
265. Puchtler, H., F. Sweat, and M. Levine, *On the binding of Congo red by amyloid.* Journal of Histochemistry and Cytochemistry, 1962. **10**: p. 355.
266. Vrana, J.A., et al., *Classification of amyloidosis by laser microdissection and mass spectrometry-based proteomic analysis in clinical biopsy specimens.* Blood, 2009. **114**(24): p. 4957-4959.
267. Rodriguez, F.J., et al., *Immunoglobulin derived depositions in the nervous system: novel mass spectrometry application for protein characterization in formalin-fixed tissues.* Laboratory Investigation, 2008. **88**(10): p. 1024-1037.
268. Thakkar, H., et al., *Development of a rapid latex enhanced turbidimetric assay for retinol binding protein in urine.* Ann Clin Biochem, 1991. **28 (Pt 4)**: p. 407-11.
269. Sanchorawala, V., *Light-chain (AL) amyloidosis: diagnosis and treatment.* Clin J Am Soc Nephrol, 2006. **1**(6): p. 1331-41.
270. Hutchison, C.A., et al., *Serum free light chain measurement aids the diagnosis of myeloma in patients with severe renal failure.* BMC Nephrol, 2008. **9**: p. 11.
271. Bridoux, F., et al., *Diagnosis of monoclonal gammopathy of renal significance.* Kidney Int, 2015. **87**(4): p. 698-711.
272. Dispenzieri, A., et al., *Appraisal of immunoglobulin free light chain as a marker of response.* Blood, 2008. **111**(10): p. 4908-15.
273. Talmud, P., et al., *Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolaemia.* Atherosclerosis, 1991. **89**(2-3): p. 137-41.
274. Gillmore, J.D., et al., *Curative hepatorenal transplantation in systemic amyloidosis caused by the Glu526Val fibrinogen alpha-chain variant in an English family.* QJM, 2000. **93**(5): p. 269-75.

275. Waldman, M., et al., *Adult minimal-change disease: Clinical characteristics, treatment, and outcomes.* Clinical Journal of the American Society of Nephrology, 2007. **2**(3): p. 445-453.
276. Nachmann, P.H., J.C. Jennette, and R.J. Falk, *Primary Glomerular Disease*, in *Brenner and Rector's, The Kidney*, B.M. Brenner, Editor. 2007, Saunders: Philadelphia.
277. Floege, J. and F. J, *Introduction to glomerular disease: clinical presentations*, in *Comprehensive Clinical Nephrology*, J. Feehally, J. Floege, and R. Johnson, Editors. 2007, Mosby/ Elsevier: Philadelphia. p. 208-218.
278. Ronco, P.M., et al., *Renal amyloidosis and glomerular diseases with monoclonal immunoglobulin deposition*, in *Comprehensive Clinical Nephrology*, J. Feehally, J. Floege, and R.J. Johnson, Editors. 2007, Mosby/ Elsevier: Philadelphia. p. 322-334.
279. Jones, B.A., et al., *Minimal Renal Amyloidosis with Nephrotic Syndrome.* Arch Pathol Lab Med, 1986. **10**: p. 889-92.
280. Brigger, D. and T.J. Muckle, *Comparison of Sirius Red and Congo Red as Stains for Amyloid in Animal-Tissues.* Journal of Histochemistry & Cytochemistry, 1975. **23**(1): p. 84-88.
281. Hetzel, G.R., et al., *AL-amyloidosis of the kidney initially presenting as minimal change glomerulonephritis.* American Journal of Kidney Diseases, 2000. **36**(3): p. 630-635.
282. Pinney, J.H., et al., *Outcome in renal Al amyloidosis after chemotherapy.* J. Clin. Oncol. , 2011. **29**(6): p. 674-681.
283. Gertz, M.A., M.Q. Lacy, and A. Dispenzieri, *Amyloidosis.* Hematol Oncol Clin North Am, 1999. **13**(6): p. 1211-33, ix.
284. Tennent, G.A., et al., *Congo red overlay immunohistochemistry aids classification of amyloid deposits*, in *Amyloid and Amyloidosis*, K.R. A and G.M. A, Editors. 1998, Parthenon Publishing: New York. p. 160-162.
285. Booth, D.R., et al., *A novel variant of transthyretin, 59Thr-->Lys, associated with autosomal dominant cardiac amyloidosis in an Italian family.* Circulation, 1995. **91**(4): p. 962-7.
286. Lachmann, H.J., et al., *Outcome in systemic AL amyloidosis in relation to changes in concentration of circulating free immunoglobulin light chains following chemotherapy.* Br J Haematol, 2003. **122**(1): p. 78-84.
287. Soutar, A.K., et al., *Apolipoprotein AI mutation Arg-60 causes autosomal dominant amyloidosis.* Proc Natl Acad Sci U S A, 1992. **89**(16): p. 7389-93.
288. Benson, M.D., et al., *A new human hereditary amyloidosis: the result of a stop-codon mutation in the apolipoprotein AII gene.* Genomics, 2001. **72**(3): p. 272-7.
289. Pote, A., et al., *Cytotoxicity of myeloma light chains in cultured human kidney proximal tubule cells.* Am J Kidney Dis, 2000. **36**(4): p. 735-44.
290. Rikitake, O., et al., *Adult Fanconi syndrome in primary amyloidosis with lambda light-chain proteinuria.* Jpn J Med, 1989. **28**(4): p. 523-6.
291. Maldonado, J.E., et al., *Fanconi syndrome in adults. A manifestation of a latent form of myeloma.* Am J Med, 1975. **58**(3): p. 354-64.
292. Sirac, C., et al., *Role of the monoclonal kappa chain V domain and reversibility of renal damage in a transgenic model of acquired Fanconi syndrome.* Blood, 2006. **108**(2): p. 536-43.
293. Sanders, P.W., *Mechanisms of light chain injury along the tubular nephron.* J Am Soc Nephrol, 2012. **23**(11): p. 1777-81.
294. Luciani, A., et al., *Impaired Lysosomal Function Underlies Monoclonal Light Chain-Associated Renal Fanconi Syndrome.* J Am Soc Nephrol, 2016. **27**(7): p. 2049-61.
295. Bonventre, J.V., et al., *Next-generation biomarkers for detecting kidney toxicity.* Nat Biotechnol, 2010. **28**(5): p. 436-40.

296. Norden, A.G., M. Lapsley, and R.J. Unwin, *Urine retinol-binding protein 4: a functional biomarker of the proximal renal tubule.* Adv Clin Chem, 2014. **63**: p. 85-122.
297. Biomarkers Definitions Working, G., *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.
298. Strimbu, K. and J.A. Tavel, *What are biomarkers?* Curr Opin HIV AIDS, 2010. **5**(6): p. 463-6.
299. Hunter, D.J., et al., *A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials.* Curr Drug Targets, 2010. **11**(5): p. 536-45.
300. Lesko, L.J. and A.J. Atkinson, Jr., *Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 347-66.
301. Puntmann, V.O., *How-to guide on biomarkers: biomarker definitions, validation and applications with examples from cardiovascular disease.* Postgrad Med J, 2009. **85**(1008): p. 538-45.
302. Lapsley, M., K. Akers, and A.G. Norden, *Sensitive assays for urinary retinol-binding protein and beta-2-glycoprotein-1 based on commercially available standards.* Ann Clin Biochem, 1998. **35 (Pt 1)**: p. 115-9.
303. Cockcroft, D.W. and M.H. Gault, *Prediction of creatinine clearance from serum creatinine.* Nephron, 1976. **16**(1): p. 31-41.
304. Gilg, J., R. Pruthi, and D. Fogarty, *UK Renal Registry 17th Annual Report: Chapter 1 UK Renal Replacement Therapy Incidence in 2013: National and Centre-specific Analyses.* Nephron, 2015. **129 Suppl 1**: p. 1-29.
305. Payne, R.B., *Renal tubular reabsorption of phosphate (TmP/GFR): indications and interpretation.* Ann Clin Biochem, 1998. **35 (Pt 2)**: p. 201-6.
306. Minisola, S., et al., *Serum ionized calcium, parathyroid hormone and related variables: effect of age and sex.* Bone Miner, 1993. **23**(3): p. 183-93.
307. Roberts, I.S., *Pathology of IgA nephropathy.* Nat Rev Nephrol, 2014. **10**(8): p. 445-54.
308. Dillon, S.C., G.M. Taylor, and V. Shah, *Diagnostic value of urinary retinol-binding protein in childhood nephrotic syndrome.* Pediatric Nephrology, 1998. **12**(8): p. 643-647.
309. Cantaluppi, V., et al., *Interaction between systemic inflammation and renal tubular epithelial cells.* Nephrology Dialysis Transplantation, 2014. **29**(11): p. 2004-2011.
310. Bougle, A. and J. Duranteau, *Pathophysiology of Sepsis-Induced Acute Kidney Injury: The Role of Global Renal Blood Flow and Renal Vascular Resistance.* Controversies in Acute Kidney Injury, 2011. **174**: p. 89-97.
311. Kyle, R.A., *Amyloidosis.* Circulation, 1995. **91**(4): p. 1269-71.
312. Wechalekar, A.D. and P.N. Hawkins, *AL amyloidosis: new drugs and tests, but old challenges.* Oncology (Williston Park), 2012. **26**(2): p. 161-2, 164.
313. Lachmann, H.J. and J.D. Gillmore, *Renal amyloidosis.* Br J Hosp Med (Lond), 2010. **71**(2): p. 83-6.
314. Algalarrondo, V., et al., *Prophylactic pacemaker implantation in familial amyloid polyneuropathy.* Heart Rhythm, 2012. **9**(7): p. 1069-75.
315. Nasr, S.H., et al., *Renal Monoclonal Immunoglobulin Deposition Disease: A Report of 64 Patients from a Single Institution.* Clinical Journal of the American Society of Nephrology, 2012. **7**(2): p. 231-239.
316. Masai, R., et al., *Clinicopathological features and prognosis in immunoglobulin light and heavy chain deposition disease.* Clinical Nephrology, 2009. **71**(1): p. 9-20.
317. Pozzi, C. and F. Locatelli, *Kidney and liver involvement in monoclonal light chain disorders.* Seminars in Nephrology, 2002. **22**(4): p. 319-330.

318. V, B. and S.B. Guan, *Receptor-mediated endocytosis of immunoglobulin light chains by renal proximal tubule cells.* American Journal of Physiology-Renal Physiology, 1997. **272**(4): p. F521-F530.
319. Ronco, P., et al., *Immunoglobulin light (heavy)-chain deposition disease: From molecular medicine to pathophysiology-driven therapy.* Clinical Journal of the American Society of Nephrology, 2006. **1**(6): p. 1342-1350.
320. Matsuzaki, K., et al., *Marked improvement by high-dose chemotherapy and autologous stem cell transplantation in a case of light chain deposition disease.* Journal of Nephrology, 2011. **24**(2): p. 246-249.
321. Pozzi, C. and F. Locatelli, *The patient with insidious chronic renal failure and the patient with the nephrotic syndrome - Two manifestations of a protean and not so rare disease.* Nephrology Dialysis Transplantation, 1996. **11**(9): p. 1876-1880.
322. Lin, J., et al., *Renal monoclonal immunoglobulin deposition disease: The disease spectrum.* Journal of the American Society of Nephrology, 2001. **12**(7): p. 1482-1492.
323. Pozzi, C., et al., *Renal-Disease and Patient Survival in Light-Chain Deposition Disease.* Clinical Nephrology, 1995. **43**(5): p. 281-287.
324. Buxbaum, J., *Mechanisms of Disease - Monoclonal Immunoglobulin Deposition - Amyloidosis, Light Chain Deposition Disease, and Light and Heavy-Chain Deposition Disease.* Hematology-Oncology Clinics of North America, 1992. **6**(2): p. 323-346.
325. Leung, N., et al., *Long-term outcome of renal transplantation in light-chain deposition disease.* Am J Kidney Dis, 2004. **43**(1): p. 147-153.
326. Short, A.K., et al., *Recurrence of light chain nephropathy in a renal allograft - A case report and review of the literature.* American Journal of Nephrology, 2001. **21**(3): p. 237-240.
327. Tovar, N., et al., *Bortezomib/dexamethasone followed by autologous stem cell transplantation as front line treatment for light-chain deposition disease.* European Journal of Haematology, 2012. **89**(4): p. 340-344.
328. Wada, Y., et al., *Light-Chain Deposition Disease Successfully Treated with Bortezomib in an Elderly Patient: A Case Report and Review of the Literature.* Internal Medicine, 2015. **54**(22): p. 2893-2898.
329. Said, S., et al., *Hypertension, renal failure, and edema in a 38-year-old man: light chain deposition disease; a case report and review of the literature.* J Nephropathol, 2014. **3**(2): p. 63-8.
330. Tennent, G.A., et al., *Congo red overlay immunohistochemistry aids classification of amyloid deposits*, in *Amyloid and Amyloidosis 1998*, R.A. Kyle and M.A. Gertz, Editors. 1999, Parthenon Publishing: Pearl River, New York. p. 160-162.
331. Kyle, R.A. and S.V. Rajkumar, *Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma.* Leukemia, 2009. **23**(1): p. 3-9.
332. Rajkumar, S.V., G. Merlini, and J.F. San Miguel, *Haematological cancer: Redefining myeloma.* Nat. Rev. Clin. Oncol., 2012. **9**(9): p. 494-496.
333. Leung, N., et al., *Monoclonal gammopathy of renal significance: when MGUS is no longer undetermined or insignificant.* Blood, 2012. **120**(22): p. 4292-5.
334. Durie, B.G., et al., *International uniform response criteria for multiple myeloma.* Leukemia, 2006. **20**(9): p. 1467-73.
335. Colombat, M., et al., *Light chain deposition disease involving the airways: diagnosis by fibreoptic bronchoscopy.* Eur Respir J, 2007. **29**(5): p. 1057-60.
336. Davies, D.F. and N.W. Shock, *Age changes in glomerular filtration rate, effective renal plasma flow, and tubular excretory capacity in adult males.* J. Clin. Invest., 1950. **29**(5): p. 496-507.

337. Comenzo, R.L. and M.A. Gertz, *Autologous stem cell transplantation for primary systemic amyloidosis.* Blood, 2002. **99**: p. 4276-4282.
338. Li, X.M., et al., *Clinicopathological characteristics and outcomes of light chain deposition disease: an analysis of 48 patients in a single Chinese center.* Ann Hematol, 2016. **95**(6): p. 901-9.
339. Yuan, Y., et al., *Urinary retinol-binding protein as a risk factor of poor prognosis in acute-on-chronic renal injury.* J Nephrol, 2016. **29**(6): p. 827-833.