Novel techniques in the diagnosis, monitoring and outcome of renal and cardiac amyloidosis

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I, Dr Tamer Rezk confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I can confirm that this has been indicated in the thesis.

Abstract

Background:

Systemic amyloidosis is a rare, progressive and fatal disorder of protein folding, treatment of which requires definitive amyloid fibril typing. Cardiac and renal involvement are common and are not only leading causes of mortality and morbidity, but also give rise to major challenges, complications and adverse effects of therapies aiming to suppress amyloid fibril precursor protein production.

Aims:

To refine and improve diagnosis, typing and management of patients with amyloidosis, through: proteomic analysis; development of novel biomarker-based risk stratification in patients with cardiorenal syndrome; evaluation of changes in body composition and cardiac dysfunction during and after systemic chemotherapy; and measures to support amyloidotic organ function.

Results and Conclusions:

I confirmed and extended previous studies demonstrating that proteomic analysis of amyloidotic tissue is superior overall to IHC for typing amyloid, but identified important limitations relating to certain tissues and amyloid types.

I characterised the complex combined cardiac and renal phenotypes that occur in one third of patients with AL amyloidosis, and performed new analyses of biomarkers that are widely used to guide chemotherapy. Very encouragingly I have shown that rapid and deep clonal responses to chemotherapy can result in meaningful improvements in patient and renal survival in individuals with advanced kidney disease, which has previously been considered beyond salvage.

Heart and kidney dysfunction both cause ECV overload and sarcopenia, which cannot be quantified clinically but contribute to poor outcomes. I used bioimpedence vector analysis to

comprehensively estimate body composition at diagnosis and to aid clinical assessment during treatment.

Despite these improvements in clinical monitoring and in chemotherapy generally, sudden cardiac death is common in amyloidosis. Sadly, ICD implantation in 15 patients described in my final chapter failed to translate into clinical benefit.

Patients with amyloidosis continue to have unmet needs, many of which stem from heart and kidney involvement, but outcomes are gradually improving.

Impact Statement

The work in my thesis focuses on diagnosis, monitoring and outcomes of patients with systemic amyloidosis. This is a rare and fatal disorder in which diagnosis is often delayed for years, associated with advanced organ failure in many cases. Despite advances in treatment, cardiac involvement remains the commonest cause of death and management and treatment of patients remains a major challenge.

The work described here has led to a number of publications and international presentations that have improved awareness of amyloidosis, along with improved diagnostic and monitoring techniques, which have resulted in improved clinical outcomes.

Comparison of traditional histological diagnostic techniques with the state-of-the-art, costly and less available mass spectrometry based proteomic analysis has provided clinicians and medical scientists an understanding of the strengths and limitations of each method and when and how they should be used in tandem, in conjunction with specialist clinical assessment.

Management and prognosis of patients with systemic immunoglobulin amyloidosis is strongly influenced by cardiac involvement using the Mayo staging system. Despite the multisystem nature of the disease, risk stratification and response to treatment is currently defined according to individual organ involvement. This is a challenge for clinicians, predominantly haematologists, in interpreting the significance of individual renal and cardiac biomarkers, in the ongoing care of such patients. The work presented in this thesis has characterised Type 5 cardiorenal syndrome in immunoglobulin light chain amyloidosis and the value of key cardiac and renal biomarkers NT-proBNP and eGFR at baseline and the following year for predicting major outcomes of death and dialysis. Additionally, whilst clonal response to treatment is well recognised as a predictor of both overall and renal survival, we show here, that in patients with advanced CKD at presentation, previously deemed to be too high risk for chemotherapy or otherwise unsalvageable, a rapid and deep clonal response is indeed beneficial resulting in a change clinical practice at our national centre and elsewhere. .

Novel work studying the role of bioimpedance vector analysis for the quantification and monitoring of body composition in patients with systemic immunoglobulin light chain amyloidosis receiving systemic chemotherapy has characterised a high incidence of sarcopenia and extracellular volume overload in patients at the time of diagnosis, which impact substantially on clinical management and overall survival. Whilst plasma NT-proBNP concentration remains the key biomarker for assessing early response to treatment, its relationship with extracellular volume overload, and its correlation with bioimpedance vector analysis provides novel information on the manner in which volume status influences NT-proBNP; this work also highlights the importance of seeking novel methods for tracking cardiac organ response in AL amyloidosis.

Lastly, whilst survival in systemic AL amyloidosis has improved substantially during the last decade, early and sudden cardiac death remains common. Intracardiac defibrillator implantation, whilst ostensibly appearing to be an attractive option, is invasive and expensive. Based upon our cohort of 15 patients who received ICD implantation and a review of the available literature, we were able to propose a pathway for patient selection for ICD implantation. Nevertheless, we sadly concluded that whilst ICDs may deliver appropriate lifesaving therapy in the short term, evidence supporting long term benefit remains unclear.

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, 09/H0715/58). The dosage and administration of radioactive isotopes were approved by the Administration of Radioactive Substances Advisory Committee of the Department of Health.

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Abbreviations

Systemic amyloid A amyloidosis	AA
Hereditary apolipoprotein AI amyloidosis	AApoAI
Hereditary apolipoprotein AII amyloidosis	AApoAII
Angiotensin converting enzyme	ACE
Amyloid enhancing factor	AEF
Atrial fibrillation	AF
Leucocyte chemotactic factor 2 amyloidosis	ALECT2
Hereditary fibrinogen A α-chain amyloidosis	AFib
Gelsolin amyloidosis	AGel
Light chain amyloidosis	AL
Hereditary lysozyme amyloidosis	ALys
Alkaline phosphatase	ALP
Autologous stem cell transplantation	ASCT
Hereditary systemic transthyretin amyloidosis	ATTRm
Wild type transthyretin amyloidosis	ATTRwt
Atrio-ventricular	AV
Bence Jones Proteins	BJP
Body Mass Index	BMI
Bioimpedance vector analysis	BIVA
Blood pressure	BP

Body Fat Mass	BFM
β-2 microglobulin	β2M
Calcium	Ca
Chronic obstructive pulmonary disease	COPD
Cerebrovascular accident	CVA
Confidence interval	CI
Chronic kidney disease	CKD
Combined liver kidney transplant	CLKT
Cardiac magnetic resonance imaging	CMR
Complete clonal response	CR
Creatinine	Creat
C-reactive protein	CRP
R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2-carboxylic acid	CPHPC
Free light chain difference	dFLC
Deoxyribonucleic acid	DNA
99mTc-3, 3-diphosphono-1, 2-propanodicarboxylic acid	DPD
Dialysis related amyloidosis	DRA
Electrocardiogram	ECG
Eastern Co-operative Group	ECOG
Ethylenediaminetetraacetic acid	EDTA
Ejection Fraction	EF

Estimated glomerular filtration rate	eGFR
End stage renal failure	ESRF
Extra cellular water	ECW
Familial amyloid polyneuropathy	FAP
Free light chain	FLC
Familial Mediterranean fever	FMF
Glycosaminoglycans	GAGs
Gamma-glutamyl transpeptidase	GGT
Gastro-intestinal	GI
Haemoglobin	Hb
Hazard ratio	HR
Hydrogen peroxide	H2O2
Implantable cardioverter-defibrillator	ICD
International Classification of Diseases	ICD-9/10
Interleukin-1	IL-1
Interleukin-6	IL-6
Intracellular water	ICW
Inter-quartile range	IQR
Iso-volumetric relaxation time	IVRT
Interventricular septal thickness in diastole	IVSd
Potassium	K
Late gadolinium enhancement	LGE

Left ventricular	LV
Left ventricular internal dimension in diastole	LVIDd
Left ventricular posterior wall thickness in diastole	LVPWd
Laser dissection mass spectrometry	LDMS
Magnesium	Mg
Monoclonal gammopathy of undetermined significance	MGUS
Monoclonal gammopathy of renal significance	MGRS
Major histocompatibility complex	MHC
Myocardial infarction	MI
Mitral valve deceleration time	MVdecT
National Health Service	NHS
UK National Amyloidosis Centre	NAC
No response	NR
Non sustained ventricular tachycardia	NSVT
N terminal pro brain natriuretic peptide	NT-proBNP
New York Heart Association Classification	NYHA
Office of national statistics	ONS
Orthotopic liver transplantation	OLT
Polymerase chain reaction	PCR
Phosphate-buffered saline	PBS
Partial response	PR
Rheumatoid arthritis	RA

Renal replacement therapy	RRT
Renal transplant	RTx
Serum amyloid A protein	SAA
Serum amyloid P component	SAP
Standard deviation	SD
Tissue Doppler imaging	TDI
Tumour necrosis factor	TNF
Total Body Water	TBW
Thyroid stimulating hormone	TSH
Transthyretin	TTR
University College London	UCL
Very good partial response	VGPR
Ventricular tachycardia	VT

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Chapter One: Introduction

The amyloidosis are a rare group of diseases that result from the extracellular distribution of amyloid, a fibrillar material derived from a range of precursor fibrils that aggregate with a highly abnormal cross B sheet confirmation (1, 2). These deposits progressively disrupt the structure of tissues and this in turn affects organ function throughout the body (3). Amyloid type is classified according to the fibril protein and there have been over 30 fibril proteins identified in vivo (Table 1.1) (4). Amyloid is identified by the pathognomonic finding of apple-green dichroism when tissue is stained with the dye Congo red and visualised under cross-polarised light. The characteristic appearance of amyloid fibrils under the electron microscope is that of rigid non-branching fibrils with a diameter of ~10 nm (5). Amyloidosis is a heterogenous condition in which the deposition of amyloid can range from incidental localised deposits in a range of different organs to a rapid and fatal systemic disease. The most commonly affected vital organs are the kidneys and heart respectively. In light of this, accurate diagnosis and a comprehensive approach to identifying organ involvement is imperative to ensure best clinical care.

Fibril Formation and Amyloid Proteins

The fibrillogenesis of amyloidosis remains poorly understood. Experiments have shown that *in vitro* under specific laboratory conditions, nearly any polypeptide chain can be misfolded with subsequent aggregation (6). Nonetheless relatively few proteins have been shown to be amyloidogenic *in vivo*.

Despite the heterogeneity of precursor proteins which form amyloid fibrils, all amyloid deposits have remarkably similar morphological structure and histological properties. The common core structure is one of anti-parallel B-strands (and less commonly parallel B-strands) that form sheets (7, 8). These B-sheets run parallel to the axis of the protofilament with their component B

strands perpendicular to the fibril axis. (9) When visualised with an electron microscope, amyloid fibrils are characteristically straight, non-branching and 7-10nm in diameter (10)

All amyloid deposits also contain numerous non-fibrillary constituents including glycosaminoglycans (GAG's), suphate proteoglycans, heparin sulphate, apolipoprotein E, type IV collagen and serum amyloid P component (SAP) (11, 12). Glycosaminoglycans are located primarily on the cell surface in the extracellular matrix and although universal to all amyloid deposits, their role remains unclear. SAP (which is identical and derived from normal plasma SAP) is another non-fibrillar part of the amyloid deposit which is bound in a reversible calcium dependant manner to a ligand present on all amyloid fibrils. It is a member of the pentraxin group of plasma proteins (13) and is resistant to proteolysis. In vitro binding of SAP to amyloid fibrils prevents degradation of amyloid by phagocytic cells and proteolytic enzymes (14). In systemic amyloidosis circulating SAP exists in a dynamic equilibrium with SAP bound to amyloid fibrils and it is only circulating SAP that undergoes catabolism. The role of SAP in amyloidogenesis has been proven by the inability to induce AA amyloidosis in SAP knockout mice (15)

There are three main circumstances in which amyloid deposition occurs. The first is in the presence of an abnormal protein with a distinctly amyloidogenic structure such as monoclonal immunoglobulin light chains in AL amyloidosis and genetic variants of transthyretin, fibrinogen Aa chain, apolipoprotein AI, apolipoprotein A2, apolipoprotein C3, apolipoprotein C2, gelsolin and lyzosyme in the hereditary causes of systemic amyloidosis. The second is when there is an abnormally high concentration of a 'normal' protein such as elevated serum amyloid A protein (SAA) in a chronic inflammatory state leading to a predisposition of AA amyloidosis or beta 2 microglobulin β2M in dialysis related amyloidosis. Lastly, amyloid deposits can occur when there is a normal quantity of a 'normal' protein that in advanced age and over a long period of time becomes amyloidogenic such as TTR in wildtype transthyretin amyloidosis (previously known as senile systemic amyloidosis).

Pathogenesis of Amyloidosis and Degradation

The mechanism of tissue damage in amyloidosis remains poorly understood. It is well known that the process of amyloid formation can result in organ dysfunction both due to a physical replacement of parenchymal tissue by amyloid deposits as well as cellular injury. There is a growing hypothesis that pre-fibrillar oligomers rather than the fibrillary form may themselves have the primary toxic effects. This has been demonstrated with the direct toxicity of the amyloidodgenic Ig light chains to cardiac cells(16) as well as transthyretin monomers in ATTR amyloidosis (17). Thus organ damage is most likely due to a combination of mechanisms including the physical disruption of amyloid on tissues and the possible toxicity of fibrils and prefibrillar aggregates which may depend on both the type of amyloidosis as well as the organ affected.

The factors that dictate the pattern of organ involvement in amyloidosis is still poorly understood. This exists both within and between different forms of amyloidosis. For example, there are clear phenotypic differences in family members within the same kindred with the same genetic mutation encoding a variant protein in hereditary forms of amyloidosis.

It is established that amyloid fibrils act as an amyloid-enhancing factor (AEF) and form a template to which further precursor proteins deposit. This theory has been supported by a study which showed that in mice, in which amyloid laden tissue from other AA amyloid-laden mice was injected, in the context of an inflammatory stimulus, the development of AA amyloidosis was markedly accelerated (18). This has been reiterated in human studies where patients who have AA amyloidosis and have a relapse of their underlying inflammatory condition rapidly deposit amyloid (19). Alongside this in patients with familial amyloid polyneuropathy (FAP) who undergo orthotropic liver transplantation (OLT) it has been shown that if cardiac amyloidosis exists pre-transplantation, cardiac amyloidosis progresses more rapidly, which is thought to be due to the enhanced deposition of wildtype TTR on a template of amyloid derived from variant TTR(20).

Untreated amyloidosis is invariably associated with progressive accumulation of amyloid often characterised by organ failure and death. Amyloid deposits are dynamic and so with adequate suppression of the precursor fibril one can see regression of amyloid from infiltrated organs (21). Studies have established that amyloid deposits are dynamic with a continuous process of amyloid formation and deposition to regression. It has been shown that macrophages play a key role in amyloid regression. Macrophages infiltrate amyloid deposits and through the formation of multinucleate giant cells which surround and engulf amyloid are key to amyloid clearance. This has been demonstrated in vivo by mouse models in which macrophage depletion with liposomal clodronate has shown to slow amyloid regression (22). The rate of clearance varies widely between individuals with some having rapid clearance of amyloid by SAP scintigraphy when there is suppression of the precursor fibril and others no regression despite complete suppression of the precursor fibril. This has led to the hypothesis that it is perhaps the different phenotype and function of macrophages between individuals that explains the heterogeneity of amyloid regression.

One striking feature of amyloid when viewed histologically is the relative absence of a cellular infiltrate or macrophages. Pepys hypothesised that coating of amyloid by SAP acts as an anti-opsonisn, further supported by the fibrillogenesis studies performed in SAP knockout mice. CPHPC, ((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexa-noyl] pyrrolidine-2 carboxylic acid), a novel bis (D-proline) drug was developed with the aim of eluting SAP from amyloid and thus promote removal of amyloid via macrophage infiltration of amyloid deposits.

Administration of CPHPC failed to rapidly elute SAP from amyloid although prolonged therapy was shown to deplete >95% of circulation SAP in the plasma and via re-equilibration, circa 90% of the SAP component of amyloidotic organs was achieved (23). Clinical studies have shown that whilst CPHPC is safe and well tolerated, the gradual depletion of SAP from amyloid deposits is insufficient to bring about major clinical benefit in visceral organ function. In mice, with systemic AA amyloidosis that are transgenic for human SAP, IgG anti-human SAP antibodies can be safely administered after treatment with CPHPC which depletes the plasma

SAP. These antibodies localise rapidly to the outstanding amyloid-bound human SAP and trigger clearance of the deposits (24). A combination of CPHPC and anti-SAP antibody has been shown in a human first into man (proof of concept) trial to result in swift and marked removal of liver amyloid deposits (25). This therapy is currently being tested in a phase 2 trial of patients with systemic amyloidosis and cardiac involvement.

Table 1.1 Classification of Systemic Amyloidosis by Precursor Protein

Fibril protein	**Precursor protein**	**Systemic and/or localized**	**Acquired or hereditary**	**Target organs**
AL	Immunoglobulin Light Chain	S, L	A, H	All organs except CNS
AH	Immunoglobulin Heavy Chain	S, L	A	All organs except CNS
AA	(Apo) Serum Amyloid A	S	A	All organs except CNS
ATTR	Transthyretin, wild type	S	A	Heart mainly in males, Ligaments, Tenosynovium
	Transthyretin, variants	S	H	PNS, ANS, heart, eye, leptomen.
Aβ2M	β2-Microglobulin, wild type	L	A	Musculoskeletal System
	β2-Microglobulin, variant	S	H	ANS
AApoAI	Apolipoprotein A I, variants	S	H	Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants)
AApoAII	Apolipoprotein A II, variants	S	H	Kidney
AApoAIV	Apolipoprotein A IV, wild type	S	A	Kidney medulla and systemic

Fibril protein	**Precursor protein**	**Systemic and/or localized**	**Acquired or hereditary**	**Target organs**
AGel	Gelsolin, variants	S	H	PNS, cornea
ALys	Lysozyme, variants	S	H	Kidney
ALECT2	Leukocyte Chemotactic Factor-2	S	A	Kidney, primarily
AFib	Fibrinogen α, variants	S	H	Kidney, primarily
ACys	Cystatin C, variants	S	H	PNS, skin
ABri	ABriPP, variants	S	H	CNS
ADan*	ADanPP, variants	L	H	CNS
Aβ	Aβ protein precursor, wild type	L	A	CNS
	Aβ protein precursor, variant	L	H	CNS
APrP	Prion protein, wild type	L	A	CJD, Fatal insomnia
	Prion protein variants	L	H	CJD, GSS syndrome, Fatal insomnia
ACal	(Pro)calcitonin	L	A	C-cell thyroid tumors
AIAPP	Islet Amyloid Polypeptide†	L	A	Islets of Langerhans, Insulinomas

Fibril protein	**Precursor protein**	**Systemic and/or localized**	**Acquired or hereditary**	**Target organs**
AANF	Atrial Natriuretic Factor	L	A	Cardiac atria
APro	Prolactin	L	A	Pituitary prolactinomas, aging pituitary
AIns	Insulin	L	A	Iatrogenic, local injection
ASPC‡	Lung Surfactant Protein	L	A	Lung
AGal7	Galectin 7	L	A	Skin
ACor	Corneodesmosin	L	A	Cornified epithelia, Hair follicles
AMed	Lactadherin	L	A	Senile aortic, Media
Aker	Kerato-epithelin	L	A	Cornea, hereditary
ALac	Lactoferrin	L	A	Cornea
AOAAP	Odontogenic Ameloblast-Associated Protein	L	A	Odontogenic tumors
ASem1	Semenogelin 1	L	A	Vesicula seminalis
AEnf	Enfurvitide	L	A	Iatrogenic

Epidemiology

Amyloidosis is a rare condition for which there is a paucity of epidemiological studies. It has been loosely estimated that 0.5-1.0 deaths per 1000 in the UK (26). The most common form of systemic amyloidosis seen in the UK is systemic AL amyloidosis with some studies suggesting an incidence of 5.1-12.8 per million person-years (27). It is estimated that approximately 800 new patients a year are referred to the NAC. Previous work at our centre has shown that the estimated minimum incidence of systemic amyloidosis in the English population in 2008 based upon new referrals to the NAC was 0.4/100,000 population. The incidence peaked at 60-79 years with systemic AL amyloidosis being the commonest type with a minimum incidence of 0.3/100,000 (28)

Non-hereditary, i.e. wildtype, transthyretin amyloidosis, which predominantly causes a cardiomyopathy in older individuals and was previously known as senile systemic/cardiac amyloidosis, is lately being diagnosed much more frequently than hitherto. This reflects the remarkable diagnostic value of cardiac MRI (CMR) and repurposing of bone scintigraphy for this indication. The true prevalence of cardiac ATTR amyloidosis remains unknown, but may be much higher than is currently apparent since post-mortem studies have long demonstrated that some ATTR deposits are present in the hearts of up to 20% of people over the age of 80 years (29). Diagnosis of wildtype ATTR amyloidosis at the NAC has risen exponentially in recent years and currently exceeds 200 patients per year.

Types of Amyloidosis

Systemic AA amyloidosis

Reactive systemic (AA) amyloidosis, in which the fibrils are composed of AA protein derived from the acute phase protein SAA, occurs as a rare complication of many chronic inflammatory disorders. The AA amyloid precursor protein is the N terminal fragment of the acute phase

reactant SAA, an apolipoprotein constituent of high-density lipoprotein. SAA is synthesized by hepatocytes and its concentration may rise 1000-fold from healthy values of less than 3 mg/L in response to inflammation. Gene transcription of SAA is regulated by cytokines, in particular interleukin (IL)-1 and IL-6.

The lifetime incidence of AA amyloidosis in patients with chronic inflammatory conditions is less than 1-5% (30). In Western Europe and the United States of America the most frequent predisposing conditions are idiopathic rheumatic diseases, notably rheumatoid arthritis and juvenile idiopathic arthritis. AA amyloidosis has become increasingly rare, reflecting improved treatment of chronic inflammatory disorders, and for reasons that are not clear, the incidence is lower in the United States than in Europe. Amyloidosis is exceptionally rare in systemic lupus erythematosus, related connective tissue diseases, and in ulcerative colitis in which there is a blunted acute phase response of SAA. Longstanding, though not necessarily constant elevation of SAA, is a prerequisite to the development of AA amyloidosis. Tuberculosis and leprosy are important causes of AA amyloidosis where these infections remain endemic. Chronic osteomyelitis, bronchiectasis, chronically infected burns, and decubitus ulcers are other well-recognized associations. Hodgkin disease and renal cell carcinoma, which often cause an acute phase response, are the malignancies most commonly associated with systemic AA amyloidosis.

Intriguingly, at least 10% of patients with AA amyloidosis do not have a clinically obvious chronic inflammatory disease, and may erroneously be assumed to have AL amyloidosis. The most common identifiable diseases found in our experience in such cases have been inherited autoinflammatory syndromes and cytokine-secreting Castleman disease tumors of the solitary plasma cell type, located either in the mediastinum or the gut mesentery. However, in the majority of these challenging patients the precise nature of the causative inflammatory disorder cannot be determined.

Autoinflammatory diseases and amyloidosis

The hereditary periodic fever/autoinflammatory syndromes are a well-described cause of AA amyloidosis, among which four are most commonly implicated. These are familial Mediterranean fever (FMF), TNF receptor-associated periodic syndrome (TRAPS), the cryopyrin-associated autoinflammatory syndrome (CAPS) and to a lesser extent mevalonate kinase deficiency (MKD) . FMF is the most common of these diseases. It is characterised by recurrent self-limiting attacks of fever, serositis and sometimes arthritis or rash (31). There is a clear ethnic preponderance of FMF being prevalent in the Eastern Mediterranean where it is the commonest genetic disease. CAPS comprises a continuous spectrum of three disorders, familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID), also known as chronic inflammatory neurological cutaneous articular syndrome (CINCA).

AA amyloidosis can complicate all types of autoinflammatory disease and whilst persistent and sustained inflammation is the key risk factor for the development of AA amyloidosis, studies have shown (predominantly in FMF) the contribution of serum amyloid A1 (SAA1) polymorphisms, differing gene mutations and birthplace. Most of these studies are small and subject to confounding influences such as increased investigation of patients who present with AA amyloidosis and more recently a significant impact from the availability of and concordance with effective long term prophylactic treatment.

SAA1 has 5 polymorphic coding alleles, SAA1.1, SAA1.2, SAA1.3, SAA1.4 and SAA1.5 (32). The gene products of these coding alleles vary by only a few amino acids at different positions of the mature SAA1 protein. Despite these minor differences, the allelic variants of SAA1 have shown differences in both in vitro assays as well as correlation with various diseases (33). The importance of polymorphisms in SAA1 differs between populations. In Japan homozygosity for SAA1.3 has been known to increase the risk of AA amyloid in rheumatoid arthritis for many years (34). In other populations with a different distribution of polymorphism, homozygosity for SAA1.1 is a risk factor. This is the case in Turkish patients

with FMF who have an increased incidence of *SAA1.1* homozygosity in FMF-amyloidosis patients (56%) compared to FMF non-amyloidosis patients (31%) suggesting a 2.5 fold increased risk (35).

MEFV M694V variant has been associated with the most severe form of FMF and a higher risk of AA amyloidosis (36). In 170 Armenian patients with FMF; 60% had a concurrent diagnosis of AA amyloidosis. The commonest genotype in this cohort was *M694V/M694V* which was present in 36% of patients and *M694V* homozygosity compared to heterozygosity was associated with an increased risk of AA amyloidosis (37).

Whilst *M694V* was previously thought to be the key risk factor for AA amyloidosis; in a large online study of 35 centres in 14 countries, 2482 cases of FMF were identified of whom 260 developed renal amyloidosis. Interestingly, country of recruitment rather than *MEFV* genotype was the leading risk factor for the manifestation of renal amyloidosis which may indicate a potential environmental origin of amyloidosis susceptibility (38).

In TRAPS the international Eurofever/Eurotraps registry identified 158 patients in whom the commonest *TNFRSF1SA* variants was R92Q (34% of cases) and T50M (10%) with disrupted cysteine residues in 27% of cases. AA amyloidosis developed in 16 (10%) patients at a median age of 43 years. This group included 7 cysteine variants (44%), two T50M (13%) and no patients with R92Q. Patients who developed AA amyloidosis had significantly longer disease duration than those who did not (39).

Retrospective literature review of all cases of CAPS from the French network for rare diseases identified 67 patients with diagnosed CAPS and AA amyloidosis. The NLRP3 gene was sequenced in 30 patients (46%) and whilst AA amyloidosis can occur in all CAPS phenotypes it appeared to be more common in MWS (Georgin-Lavialle; 2015; Paediatric Rheum Online).

It is important to recognise that these genetic associations are weak and not useful predictors of risk in individual patients. The remarkably high risk of AA amyloidosis in systemic auto inflammatory diseases reflects persistent and uncontrolled inflammation. Although small series have identified increased risks with specific genotypes in FMF and perhaps with mutations affecting cysteine residues in TRAPS there are no guaranteed ‘safe’ mutations and

all patients should be treated to completely supress chronic inflammatory disease and SAA production. The same point is true for the data on SAA polymorphisms and cases of AA amyloidosis.

Clinical features of AA amyloidosis

AA amyloid involves the viscera, but may be widely distributed without causing symptoms. It predominantly affects the kidneys with more than 95% of patients presenting with proteinuria and around 10% having already reached end-stage renal failure (ESRF) at diagnosis (19).

The predominant presentation is nephrotic syndrome with non-selective proteinuria from glomerular deposition of amyloid and or chronic kidney disease. Splenic involvement is evident on SAP scintigraphy in almost all cases and whilst deposits commonly occur in the adrenal gland and gastrointestinal tract, this is usually without associated organ dysfunction. Liver involvement in AA amyloidosis is a feature of advanced disease and confers a poor prognosis (40). Cardiac amyloidosis and amyloid related neuropathy are rare manifestations of AA amyloidosis and are seen only in advanced cases.

Systemic AL amyloidosis

This is the most common form of amyloidosis in the developed world is associated with dyscrasias of cells within the B lymphocyte lineage, including multiple myeloma (MM), malignant lymphomas and macrogloublinemia. Most cases develop in the context of what would otherwise be a low grade monoclonal gammopathy of unknown significance (MGUS). The age adjusted incidence in the USA is 8.9 per million person-years (27). Amyloidosis occurs in up to 10% of cases of MM and in a lower proportion of other malignant B-cell disorders. Approximately 2% of patients with an MGUS eventually develop AL amyloidosis (27). The fibrils are formed from the N terminal domain of monoclonal lambda (more common) or kappa immunoglobulin light chains, and consist of the whole or part of the variable (VL) domain.

A monoclonal immunoglobulin can be detected in the serum or urine by immunofixation electrophoresis in 65% and 86% of patients, respectively. A monoclonal excess of free light chains (FLC) can be identified at baseline in 98% of patients with systemic AL amyloidosis. Subnormal levels of some or all serum immunoglobulins, or increased numbers of marrow plasma cells may provide less direct clues to the underlying etiology. Until recently, it has been the practice to consider apparent primary cases of amyloidosis, with no previous predisposing inflammatory condition or family history of amyloidosis, as AL type. However, it has now been recognized that some patients with mutations associated with autosomal dominant hereditary non-neuropathic amyloidosis, particularly that caused by variant fibrinogen α-chain, do not develop the disease,. The coincident occurrence of a monoclonal gammopathy, which occurs in more than 10% of the healthy older population, may then be gravely misleading and it is essential to exclude other forms of amyloidosis by genotyping all known amyloidogenic mutations, and to seek definitive immunohistochemical or proteomic identification of the amyloid fibril protein in all cases.

Clinical manifestations of AL amyloidosis

Clinical suspicion of AL amyloidosis should be raised in any patient with unexplained nephropathy, cardiac failure, peripheral and/or autonomic neuropathy or any other multisystem disease. Potentially all organs can be directly affected by amyloid deposits in systemic AL amyloidosis except the central nervous system. Renal involvement is the most common manifestation with approximately 70% of patients presenting with either proteinuria or elevated serum creatinine. Cardiac amyloidosis is present in 50% of patients at baseline and is the key determinant of mortality. Cardiac amyloidosis typically manifests with a restrictive cardiomyopathy; concentric ventricular wall thickening resulting in diastolic dysfunction manifesting with congestive cardiac failure and, more often than not, hypotension. Autonomic nervous system involvement presents variably and is often challenging to diagnose. It can lead to orthostatic hypotension, erectile dysfunction, urinary retention and faecal incontinence. In patients in whom a peripheral neuropathy is present

there is most commonly a distal sensory deficit which can be subclinical at presentation. Systemic chemotherapy aimed at suppressing the monoclonal light chain can cause worsening of peripheral and autonomic neuropathy depending upon the neurotoxicity of therapy. Liver involvement as a presenting feature is rare but is a quite common finding at post mortem examination and on SAP scintigraphy. Hepatomegaly and obstructed liver function tests are the most common clinical findings but can be absent in patients despite the presence of significant hepatic amyloid deposits (41).

There are a plethora of soft tissue features in AL amyloidosis with macroglossia and periorbital bruising thought to be highly suspicious for AL type. Gastrointestinal involvement can result in malabsorption, altered bowel habit and gastrointestinal haemorrhage (42).

Hereditary systemic amyloidosis

Familial amyloid polyneuropathy

Familial amyloid polyneuropathy (FAP) is associated with more than 100 mutations in the gene encoding TTR. TTR is predominantly synthesized in the liver and is a tetrameric protein which has a role in the transport of thyroxine and retinol binding protein. FAP is an autosomal dominant syndrome with onset of symptoms at any point from the second decade onwards. It was first described in 1952 in Portuguese kindreds (43). It is characterised by progressive peripheral and autonomic neuropathy alongside varying involvement of visceral organs. Extra-neural manifestations predominantly include cardiomyopathy as well as more rarely vitreous amyloid, renal involvement and oculoleptomeningeal amyloid deposition leading to encephaolopathy, seizures and dementia. The combination of neuropathy and cardiomyopathy leads to muscle wasting and malnutrition that usually results in death within 9-13 years (44). The most common encoding mutation is a valine for methionine substitution at position 30 (V30M) and

quite large populations occur in Sweden, Japan and Portugal. The T60A variant is most common in the UK, and the low penetrance V122I variant associated with predominant cardiomyopathy occurs in 3-4% of black individuals. Proposed mechanisms of ATTR amyloidogenesis include dissociation of the TTR tetramer into monomers and mechano-enzymatic cleavage with resulting destabilising of the tetrameric TTR protein (45).

Non neuropathic systemic amyloidosis

The non-neuropathic forms of hereditary systemic amyloidosis were first described in 1932 and are derived from variants of apolipoprotein AI, apolipoprotein AII, lysozyme and fibrinogen A- α chain. Renal involvement is often the most common manifestation however the heart, spleen, liver and bowel may all also be involved. Presentation can vary both within and between kindreds. Clinical presentation is usually around the sixth decade although can occur in early adulthood or before. Following clinical presentation, there is an inexorable progression to organ failure requiring dialysis, organ transplantation or death. In fibrinogen A- α chain amyloidosis the median time from presentation to end stage renal disease (ESRD) is approximately 5 years (46). The progression of renal disease is much more gradual in apolipoprotein AI and lysozyme amyloidosis with a median time from presentation to ESRD of greater than 10 years.

Wildtype transthyretin amyloidosis (previously known as senile systemic/cardiac amyloidosis)

Wild type transthyretin amyloidosis (ATTRwt) also known as senile systemic/cardiac amyloidosis is a disease of older people with a strong male preponderance. The amyloid deposits are composed of wildtype TTR (47). The clinical phenotype comprises predominantly of cardiac amyloidosis manifesting as congestive cardiac failure. ATTR deposits are present in other sites including the lungs, gut and bladder, where they can

occasionally cause symptoms (48). Carpal tunnel syndrome is common and often precedes cardiac manifestations by up to a decade or more (49).

Diagnosis and Assessment of organ function

Histology

The diagnosis of amyloidosis is often made at a late stage in the disease process, long after the initial onset of symptoms. Delays in diagnosis are due to a combination of the heterogeneous nature of the disease, its perceived rarity and the need for staining of histological sections. The gold standard for diagnosis is the histological confirmation of amyloid deposits by Congo-red staining and observing the pathognomonic apple green birefringence under cross polarised light visualised by light microscopy (50). Correct typing of amyloid deposits is crucial to identify the pathological precursor fibril and key to deciding disease appropriate treatment with suppression of the protein precursor fibril. Incorrect diagnosis and management can lead to significant morbidity and even mortality to the patient (51).

Congo red staining has an estimated sensitivity of between 60–80% with a specificity of 100% (52, 53). Target organ biopsies such as renal, cardiac and GI tissue are usually diagnostic. Rectal biopsies have been used in the past as a screening tool for systemic amyloidosis with estimated sensitivity rates of 75-94% in published series (54). Abdominal fat pad fine needle aspiration is a quick, minimally invasive bedside test that has historically been thought to yield poor sensitivity in systemic amyloid (55). The role of fat aspiration is particularly pertinent where target organ biopsies (such as endomyocardial biopsies) require technical expertise and carry with them a risk of serious complications and high cost, often leading to a delay in diagnosis and treatment. We have shown that abdominal fat aspiration can be useful in detecting cardiac amyloidosis with a sensitivity that ranges from 84% in patients with cardiac AL amyloidosis to 15% in those with wildtype ATTR cardiac amyloidosis (56).

Determining the fibril precursor protein

The gold standard method for identifying the amyloid fibril protein is via direct sequencing of extracted amyloid. The problem with this technique is the time, cost and necessity for frozen tissue. For this reason it is not used in routine clinical practice. Alternative methods for the diagnosis of amyloid and typing include identifying the amyloid fibril protein by immuno-electron microscopy (57) but again this method is not widely available, expensive and rarely employed in clinical practice.

Immunohistochemical staining (IHC) of amyloidotic tissue is widely available for determining the amyloid fibril protein. It is a relatively quick procedure and is the preferred method in clinical practice (50). IHC in routine clinical practice has variable sensitivity and specificity however it is well known that amyloid deposits can sometimes fail to stain or stain with more than one antibody. For example, work from our centre has shown that in approximately 30% of patients with systemic AL amyloid fail to stain definitively with antibodies against either kappa or lambda light chains (58).

Proteomics and Mass Spectrometry

Proteomic analyses comprising mass spectrometry on amyloid deposits cut by Laser dissection of tissue sections is increasingly used to identify the presence of amyloid and subtype. Some centres estimate between 98% to 100% specificity and sensitivity (59). It is increasingly used for the typing of organ specific biopsies (60). The role of proteomics in identifying amyloid from fats has been reported. It has been estimated that in Congo red positive samples, the sensitivity of identifying the amyloid subtype is up to 90%(61). Whilst other amyloid centres have not necessarily had the same success with proteomics, it has undoubtedly aided in the typing of amyloid.

One of the limitations of proteomics in the subtyping of amyloid is the detection of more than one potentially amyloidogenic protein, especially immunoglobulins and transthyretin which are

abundant plasma proteins. We have shown here in our centre that in cases where more than one amyloidogenic protein is detected, decellularisation of amyloid tissue biopsies can increase the accuracy of proteomic typing and enhance the specificity of detecting the culprit protein (62).

Genetic Sequencing

It is estimated that between 5-10% of systemic amyloidosis is hereditary. Genetic testing is often key to identifying the amyloid subtype but needs to be interpreted in clinical context. Variants within genes encoding amyloidogenic proteins such as TTR can present with distinctly different phenotypes as both the penetrance is variable and incidental mutations can be identified. This is particularly relevant in systemic AL amyloidosis where treatment is aimed at suppression of the underlying monoclonal protein and incidental mutations can lead to a delay in treatment (63). Conversely, particularly in an aging cohort, the incidence of an MGUS has been estimated at over 5% in patients aged greater than 70 years and 7.5% in those 85 or older (64). In the context of hereditary amyloidosis this has the potential for misleading clinicians and the inappropriate delivery of cytotoxic therapy (51)

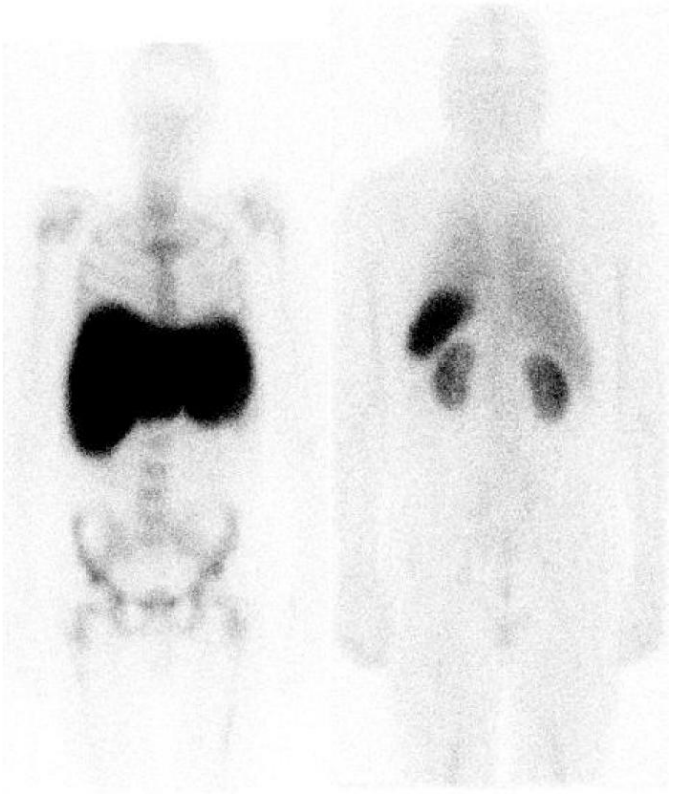
Imaging

Serum Amyloid P (SAP) Scintigraphy

SAP scintigraphy is a specialised imaging technique developed by Professor Hawkins at the NAC, available in a limited number of specialist centres. SAP is a non-fibrillar part of the amyloid deposit which is thought to be present in all amyloid deposits and makes radiolabelled SAP scintigraphy specific(65). Following intravenous injection, radiolabelled SAP tracer can be used to identify amyloid deposits in vivo. It identifies amyloid deposits in visceral organs such as the spleen, liver, kidneys and adrenal glands (65). Organ involvement by SAP scintigraphy

can be pathognomonic of amyloid subtype. For example, bone uptake in the context of clinical suspicion is almost always diagnostic of AL amyloidosis (Figure 1.1).

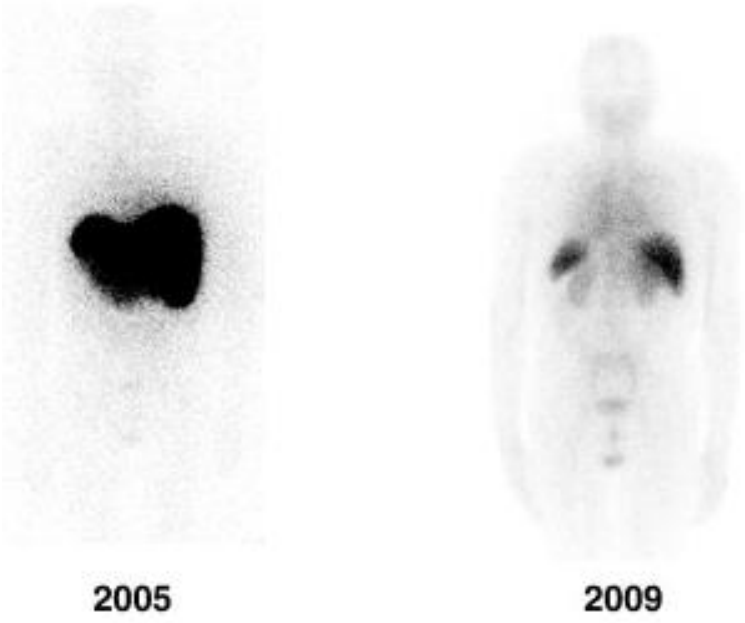
Figure 1.1 (Left) Anterior whole body scintigraphic image following intravenous injection of ^{123}I-human serum amyloid P in a patient with AL amyloidosis. Uptake is seen in the bones, a finding which is pathognomonic for AL amyloidosis, uptake is also seen in the liver and spleen. (Right) Posterior whole body SAP scintigraphic image in a patient with hereditary fibrinogen amyloidosis. Uptake is seen in the spleen and kidneys.



There are limitations to SAP scintigraphy in light of its methodology. Due to the significant pool and movement of blood in the lung and hearts it is not adequate for identifying cardiac or pulmonary amyloid deposits (66). Alongside this, there is insufficient resolution to identify deposits in hollow, diffuse or very small structures such as the GI tract, skin and nerves.

SAP scintigraphy has been used to monitor the progression and regression of visceral amyloid deposits and can inform clinicians about response to treatment. It shows the dynamic nature of amyloid deposits and proves to be a useful tool in detecting and monitoring the response to treatment and amyloid regression (25, 65)

Figure 1.2: Posterior whole body images of SAP scintigraphy scans of a patient with systemic monoclonal immunoglobulin type (AL) amyloidosis who presented with major liver involvement and proteinuria in 2005. He responded well to chemotherapy with substantial regression of amyloid by 2009 when his liver and renal function had returned to normal.



Unfortunately due to the high cost of labelling and the availability of SAP and I^{123}, the use of SAP scintigraphy has been limited to a few European centres of which the National Amyloidosis Centre has completed over 50,000 scans.

Cardiac Imaging

Cardiac involvement is the key predictor of mortality in systemic amyloidosis (67, 68). Non-invasive diagnosis of cardiac amyloidosis has historically been based on imaging modalities such as transthoracic echocardiography. This classically demonstrates interventricular septal diameter (IVSD) thickening and restrictive diastolic physiology. In systemic AL amyloidosis cardiac involvement is defined as a mean left ventricular diameter of (LVD) >12mm in the absence of an alternative cause of left ventricular hypertrophy (69). Nonetheless there are limitations in the use of echocardiography for the diagnosis of cardiac amyloidosis with studies showing both a poor sensitivity and specificity, particularly in differentiating cardiac amyloidosis from other causes of thickened LVD such as hypertrophic cardiomyopathy and hypertensive heart disease (70).

Cardiac magnetic resonance imaging (CMR) for cardiac amyloidosis was first reported in 2005 and is now increasingly used in clinical practice to diagnose infiltrative cardiomyopathies. CMR demonstrates characteristic late gadolinium enhancement (LGE) in the sub endocardium or in some cases more diffusely (71). CMR has been shown to be more informative of both the structural and functional changes seen in cardiac amyloidosis than other imaging modalities (72). For example in ATTR cardiac amyloidosis CMR demonstrated asymmetrical hypertrophy as the commonest pattern of ventricular remodelling and although other morphologies were possible, all patients had evidence of characteristic LGE. (73)

Cardiac Rhythm Analysis

Electrocardiographic changes are common in cardiac amyloidosis. The largest study consisted of 127 patients with biopsy proven cardiac AL amyloidosis seen at the Mayo clinic and this confirmed a characteristic appearance of low QRS (limb leads <5mm) with poor R wave progression in the chest leads in approximately 50% of patients (74). Cardiac rhythm analysis is

particularly pertinent in patients with cardiac AL amyloidosis due to the very high early mortality seen, often within the first few months of diagnosis (30-40%) despite significant improvement in treatment regimens (75). The main cause of this early mortality is sudden cardiac death (SCD). The prevalence of ventricular arrhythmias has been estimated at up to 30% of patients with cardiac AL amyloidosis (76) with both ventricular tachyarrhythmias (77) and bradyarrhythmias reported. Work from our centre using loop recorders showed that in 20 patients with advanced cardiac AL amyloidosis (Mayo Stage 3b) disease the predominant abnormality detected on telemetry was bradycardias, most commonly complete heart block (CHB) and this heralded terminal cardiac decompensation in most patients (78)

Cardiac ATTR amyloidosis is also associated with significant abnormalities on ECG. Low QRS voltages are less frequently described in cardiac ATTR estimated at 25-40% (74, 79) however a pseudo infarct pattern is seen as commonly as in cardiac AL amyloidosis. The presence of conduction system disease is more common in patients with wildtype cardiac ATTR; with atrial fibrillation (AF) the most commonly noted rhythm disturbance in approximately 40% of patients with cardiac ATTR.(80)

Biochemical Analysis

Investigations for Clonal Disease

AL amyloid fibrils are composed of monoclonal immunoglobulin light chains driven by an underlying clonal dyscrasia which may be very subtle. In all patients in whom systemic amyloidosis is suspected a full clonal work up should be performed. Monoclonal proteins can be detected by serum and urine electrophoresis and immunofixation. Serum free light chains should be tested in all patients with suspected systemic amyloidosis and it has been shown that fully quantitative high sensitivity serum free light chain immunoassay (Freelite) has improved the sensitivity of detection of an underlying clone (81). Recent data indicates that in approximately 1% of patients with systemic AL amyloidosis, no systemic clone is identified (82) which can make both diagnosis and the monitoring of response to treatment particularly challenging. The

clonal dyscrasia in patients with systemic AL amyloidosis can vary from being extremely low to frank multiple myeloma (MM). All patients therefore should undergo routine myeloma workup including a bone marrow trephine and aspirate in order to assess the baseline plasma cell infiltrate and flow cytometry for cytogenetics which may exist and inform outcome (83). The translocation t11:14 is thought to be present in up to half of patients with systemic AL amyloidosis and less so in 15% of patients with MM (84). Whilst skeletal X-rays were previously commonly used for the detection of lytic lesions, monitoring of bone lesions in MM is better performed by MRI or increasingly Whole-body low-dose CT scan (85). Accurate diagnosis of the plasma cell burden, correct cytogenetic profiling and assessment for multiple myeloma in patients with systemic AL amyloidosis is important for both prognosis and treatment options.

Cardiac Biomarkers

The two biomarkers used routinely in the diagnosis and monitoring of patients with cardiac amyloidosis are N terminal pro brain naturetic peptide (NT-proBNP) and highly sensitive cardiac troponin T (TnT). Both are part of the most widely used staging systemic for cardiac AL amyloidosis, the Mayo classification (Table 1.2) (86). Mayo classification has provided key information on overall survival (OS) in patients with cardiac AL amyloidosis with an estimated median survival of 27.2 months, 11.1 months and 4.1 months in Mayo Stage I, II and III respectively. Further sub classification of Mayo Stage III disease into IIIa and IIIb is used to identify patients at very high risk of early mortality based upon the presence of systolic dysfunction defined as either systolic BP <100mm/Hg and/or NT-proBNP of >8500ng/L (68). Both NT-proBNP and cardiac troponin T can be elevated due to a number of other conditions including atrial fibrillation, pneumoniae and renal failure (87). The limitations of NT-proBNP in patients with systemic AL amyloidosis and advanced renal excretory impairment in systemic AL amyloidosis have been noted (88). NT-proBNP is also known to be influenced by systemic chemotherapy (89) as well as fluid status. (90). NT-proBNP is specifically relevant as it is the key determinant of a response to treatment in systemic AL amyloidosis as per current

consensus criteria. (91) Cardiac ATTR amyloidosis is an increasingly recognized and fatal cardiomyopathy and whilst it has been historically felt to have a slowly progressive nature, the natural history can vary significantly. Recent work from our centre has identified a new staging system for ATTR cardiac amyloidosis based upon a combination of baseline NT-proBNP and eGFR. Three disease stages were identified; Stage 1 was defined as NT-proBNP ≤ 3000ng/L and eGFR ≥ 45ml/min, Stage 3 as an NT-proBNP of ≥ 3000ng/L and eGFR ≤ 45ml/min and the remainder stage 2. Median survival in Stage 1, Stage 2 and Stage 3 disease was 69, 46 and 24 months respectively. (80)

Table 1.2 Mayo Staging in cardiac AL amyloidosis

Mayo Stage	**Cardiac Biomarkers**
Stage 1	NT proBNP < 332ng/L and cardiac troponin T < 0.035mcg/L
Stage 2	NT proBNP ≥ 332ng/L or cardiac troponin T ≥ 0.035mcg/L
Stage 3	NT proBNP ≥ 332ng/L and cardiac troponin T ≥ 0.035mcg/L

Renal Biomarkers

Renal involvement in amyloidosis typically manifests with proteinuric CKD, often associated with nephrotic syndrome. Nonetheless the degree of proteinuria can be variable both between and within different types of renal amyloidosis dependant on the amyloid fibril protein as well as stage of CKD and/or urinary output.

The three key biomarkers for the diagnosis and prognostication of patients with renal amyloidosis include serum albumin, proteinuria and serum creatinine/estimated glomerular

filtration rate (eGFR). Consensus criteria defines renal involvement in systemic AL amyloidosis as non Bence Jones (BJP) proteinuria of >0.5g/24hrs (91). Both renal progression and renal response to treatment is dependent upon improvement or worsening or proteinuria in the context of a change in eGFR. In light of the need for improved prognostication of the risk of requirement for renal replacement therapy in renal AL amyloidosis, revised criteria has been proposed by Palladini et al which demonstrated that a ≥ 25% reduction in eGFR at an earlier 6 month time point predicted a poor renal survival.

The monitoring of renal amyloidosis based on proteinuria has its limitations in light of alternative pathologies that can drive urinary protein leak, including diabetes and hypertension. Novel urinary biomarkers have been used in monoclonal gammopathies of renal significance (MGRS) to detect renal insult and may offer improved methods of both diagnosis and monitoring in renal amyloidosis (92).

Liver function tests

Despite significant amyloid deposit in the liver displayed by SAP scintigraphy, liver function is often preserved in patients with systemic amyloidosis. Whilst the liver seems to tolerate amyloid deposits relatively well, it has been shown in systemic AL amyloidosis that an elevated bilirubin is associated with an increase in relative risk of almost 2.5 (93)

General management principles

There are three key principles in the management of systemic amyloidosis. Supportive care to preserve organ function, reduction, or ideally elimination, of the ongoing supply of the respective amyloid fibril precursor protein, and relatively novel therapies aimed at inhibiting the formation of amyloid fibrils or removing existing amyloid deposits.

Supportive care

Best supportive care is vital for patients with all forms of systemic amyloidosis. The aim is to support failing amyloidotic organ function and reduce the risk of complications in vulnerable organs.

Kidneys extensively infiltrated by amyloid are exquisitely vulnerable to intercurrent insults such as hypo/hyper perfusion and nephrotoxic drugs which should be avoided as much as possible. The management of nephrotic syndrome includes meticulous fluid balance encouraging patients to pursue a low salt diet in combination with a total fluid restriction of 1.5 litres per day. Diuretic therapy is the mainstay of medical management and loop diuretics are often required at high doses and/or in combination with either thiazide or potassium sparing diuretics (94). Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) have been shown to reduce proteinuria and reduce the risk of progression to ESRD in patients with nephrotic syndrome (95). Nonetheless due to their risk of acute kidney injury they are often not used in the initial setting. One study of 44 patients with systemic AA amyloidosis showed a reduction in proteinuria in patients treated with ARB although there was no clear long term benefit in reducing the risk of progression to ESRD (96).

Anticoagulation in nephrotic syndrome remains a controversial issue with no clear consensus. Patients with systemic amyloidosis have an increased risk of bleeding due to amyloidotic vascular fragility whilst patients with heavy proteinuria and associated hypoalbuminemia are at an increased risk of venous thromboembolic disease. Decisions regarding anticoagulation should be made on an individual basis and in high risk patients low molecular weight heparin

treatment can offer a suitable option with a short half-life and easy reversal compared to warfarin therapy.

Management of cardiac amyloidosis is challenging. Patients with cardiac amyloidosis do not tolerate hypotension well due to the low cardiac output state and whilst there are no clear guidelines on the use of traditional heart failure medication, ACE inhibitors and β-blockers are generally best avoided. Arrhythmias are common in AL amyloidosis. The mainstay of therapy is with oral anti-arrhythmias, most commonly amiodarone. There are limited data on the role of implantable intracardiac defibrillators in patients with cardiac amyloidosis. Appropriate device therapy has been reported in a significant proportion of patients and whilst it has been shown to be lifesaving in the short term, long term survival benefit remains unclear (97).

Gastrointestinal involvement can present with chronic diarrhea, malabsorption and cachexia with symptoms often becoming debilitating. Gastrointestinal bleeding can occur manifesting with melena and anemia. Somatostatin analogues have provided relief in some case studies, but if symptoms lead to malnutrition total parenteral nutrition may be needed to support the patient until bowel function improves.

Adrenal involvement in AA amyloidosis is common but frank adrenal insufficiency is rare. Patients are often receiving corticosteroid therapy for their underlying inflammatory condition. Addisonian symptoms can be difficult to identify, particularly orthostatic hypotension which can be explained by alternative pathologies such as nephrotic syndrome and concurrent diuretic use.

Amyloid related autonomic nerve dysfunction is predominantly seen in AL amyloidosis and hereditary ATTR amyloidosis. Autonomic failure often manifests predominantly with postural hypotension but other symptoms include altered bowel habit, incontinence and erectile dysfunction. Anecdotal evidence supports the use of oral inotropes such as midodrine for the treatment of postural hypotension.

Organ transplantation in hereditary amyloidosis

Disease modifying treatment for hereditary systemic amyloidosis remains limited. The mainstay of treatment is supportive therapy including organ transplantation for the failing amyloidotic organ. When the liver is the main source of production of variant precursor protein, liver transplantation can be performed to replace the variant protein with the wildtype non amyloidogenic protein.

Fibrinogen, TTR and ApoA1 are predominantly synthesised in the liver. Whilst liver transplantation can be been lifesaving in selected patients with hereditary amyloidosis, careful consideration needs to be taken due to the considerable peri-operative risk, long term immunosuppression, renal toxicity and development of secondary malignancies.

Hereditary AFib amyloidosis is a predominantly renal disease leading to ESRD within 5-10 years. Kidney transplantation has been performed in many cases, but recurrence of renal amyloidosis within 7-10 years commonly causes graft failure. Whilst combined liver-kidney transplantation eliminates the source of the amyloidogenic AFib variant, with potential to prevent further amyloid deposition, the procedure is associated with some mortality and is best reserved for younger patients (98).

Treatment of AA amyloidosis

Treatment depends on the nature of the underlying chronic inflammatory disorder and ranges from potent anti-inflammatory and immunosuppressive biological drugs in patients with rheumatoid arthritis, to lifelong prophylactic colchicine in FMF and surgery in conditions such as refractory osteomyelitis and the cytokine secreting tumors of Castleman disease.

Most patients with AA amyloidosis complicating inflammatory arthritis can now be treated effectively with one or other of the many biological agents now available, i.e. anti-cytokine (TNF, IL-1, IL-6) and anti-CD20 antibodies. Nonetheless whilst there have been advances in the use of biologic therapies, progressive renal dysfunction remains common in AA amyloidosis and the need for renal replacement therapy occurs in up to a 40% of patients with a median time to dialysis from diagnosis of 6.5 years. Mortality, amyloid burden and renal prognosis are all

significantly correlated with SAA concentration during follow up. In a study of 374 patients with systemic AA amyloidosis, the risk of death was 17.7 times higher in patients with SAA concentrations ≥155 mg/L compared to <4mg/L. In fact even in patients with AA amyloidosis and modestly elevated SAA levels (4-9 mg/L), the risk of death was four fold higher compared to those with SAA of <4 mg/L.(19). Complete suppression of inflammation (SAA concentration persistently <4 mg/L) is frequently associated with gradual regression of amyloid and preservation of renal function (19).

Colchicine (in FMF) at the maximum tolerated dose and IL-1 inhibition with biological agents has revolutionised the management and prognosis of many patients with inherited autoinflammatory/periodic fever syndromes. Work from the National Amyloidosis Centre in AA amyloidosis complicating hereditary periodic fever syndromes has shown that that the diagnosis of a hereditary periodic fever syndrome had not been considered in half of patients prior to presentation with AA amyloidosis, almost 25% had evidence of ESRD at presentation and a further 28% developed ESRD over the course of follow up with a median time of 3.3 years. Of the 46 patients assessed, 24 had FMF, 12 TRAPS and 6 CAPS. The majority of patients with FMF (22/24) were treated with high dose colchicine with complete remission in 19 and partial remission in 1 of the underlying inflammatory condition. Of the 12 patients with TRAPS, 6 patients were initially treated with anti-TNF therapy with a transient response seen in 4; all switched to IL-1 blockade. 4 patients were treated upfront with IL-1 blockade. Of the 6 patients with CAPS, 4 were treated with IL-1 blockade with dramatic clinical and laboratory improvement and 2 died before the role of IL-1 therapy in CAPS was recognised. Of the total 37 patients from the cohort who were treated successfully, or in whom at least partial suppression of the underlying autoinflammatory condition was achieved, 17 (46%) showed amyloid regression, 14 (38%) showed a stable amyloid load, and 2 (5%) showed increased amyloid deposition over the follow up period (99).

The preferred form of RRT remains renal transplantation and suppression of the underlying inflammatory disorder is imperative prior to transplantation. In a study looking at renal transplantation in 128 patients with AA amyloidosis and ESRF, 43 underwent renal

transplantation with a median time from ESRF to transplantation of 1.5 years. Median estimated graft survival noncensored for death was 10.3 years; with 5 and 10 year graft survival of 86% and 59% respectively. 16 (37%) of patients died most commonly from infection and median SAA levels were higher in patients with recurrent amyloid in the graft compared to those in whom amyloid did not recur (100).

Treatment of AL amyloidosis

The current management of AL amyloidosis is aimed at suppressing the underlying B cell clone as quickly and completely as possible with chemotherapy and novel agents. This in turn halts the production of amyloidogenic light chains. Remission of the underlying clonal disease, i.e. hematological responses, may be associated with preservation of organ function and in some cases improvement in organ function, i.e. organ responses, especially when hematological remission has been sufficient to facilitate some gradual regression of the amyloid deposits.

Consensus criteria to define hematological response and organ responses in AL amyloidosis have been devised (91). Patients who achieve a complete hematological response have the best clinical outcomes (101). This is defined by no detectable monoclonal immunoglobulin [M] band in serum or urine by immunofixation and normal free light chains, or a very good partial response, defined as the difference between the involved and uninvolved light chains (dFLC) <40mg/L.

Although chemotherapy for AL amyloidosis has very largely been adapted from substantial experience in MM, adverse effects of treatment in patients with amyloidosis are much more frequent and serious, due to the reduced functional reserve of amyloidotic organs and poor performance status of many patients. This has led to risk adapted chemotherapy protocols, with most AL amyloidosis patients being classed as intermediate risk and best suited to cyclic combination chemotherapy regimens. These have historically included oral melphalan with dexamethasone (MDex) as well as a combination of cyclophosphamide, thalidomide and dexamethasone (CTDa). More lately, proteasome inhibitors, initially bortezomib and now others, have become the cornerstone of treatment (102). First line combination therapy with

bortezomib, cyclophosphamide and dexamethasone (CyBorD) has been shown to deliver high overall response rates (103). Ixazomib is the first oral proteasome inhibitor and is available in combination with lenolidamide and dexamethasone after at least one prior line of therapy. Carfilzomib is a novel irreversible proteasome inhibitor approved for relapsed/refractory MM. Whilst Phase I/II studies of its use to treat systemic AL amyloidosis have shown promising hematological response rates, cardiac, renal and pulmonary toxicity have been noted, warranting close monitoring of side effects and dose reduction.

Autologous stem cell transplantation (ASCT), both in the initial and relapsed disease settings, is an effective treatment for AL amyloidosis leading to deep and durable clonal responses with an excellent median overall survival of over 5 years. However, this high intensity treatment is suitable for only a minority of patients due to significant procedure related morbidity and mortality (104). Stringent risk stratification has helped improve outcomes, and use of Mayo cardiac staging criteria has resulted recently in procedural mortality rates of 7% or less (104).

Median survival in AL amyloidosis has improved a great deal over the past decade with a current estimated 4 year survival rate of 50%. Sadly, nearly 25% of patients still die from disease related complications within the first few months of treatment and this is primarily due to the presence and severity of cardiac involvement (75).

Novel therapeutic approaches in clinical trials

A number of different therapies aimed specifically at inhibiting the formation of amyloid fibrils or promoting fibril regression are currently under development, and some have already being clinically evaluated.

In-vitro studies have shown that amyloidogenic misfolding of TTR may be inhibited by compounds that bind TTR in the plasma. Tafamadis, which is a TTR stabiliser, has been developed specifically to treat ATTR amyloidosis and to slow neuropathic disease in patients with V30M familial amyloid polyneuropathy (105). Diflunisal, a non-steroidal anti-inflammatory drug, has lately been repurposed as an amyloid treatment, unrelated to its anti-inflammatory properties; it also binds to and stabilises TTR in vitro (106). A randomized

controlled trial confirmed that it slows neurological progression in hereditary ATTR amyloidosis (107). TTR is almost exclusively synthesized by the liver which presents a target for state of the art RNA-inhibiting therapies. Anti-sense oligonucleotide and small interfering RNA therapies have been shown to reduce circulating TTR by 70-85% respectively (108), and phase 3 studies have lately been completed of both class of agents with great success, showing substantial inhibition and even reversal of neuropathic features in FAP (109).

Anti-amyloid antibodies

The role of therapeutic antibodies to directly target existing amyloid deposits is being investigated with vigor. There are currently three monoclonal antibodies that are undergoing testing.

The first antibody approach focuses on the murine monoclonal 11-1F4 antibody prepared against human light chain related fibrils and recognised an amyloid-associated conformational epitope (110). In animal models of mice bearing human amyloidomas, rapid and complete elimination of the masses without toxicity was demonstrated. In an open label, dose escalation phase I clinical trial, the drug was tolerated well by participants with no grade 4 or 5 adverse events reported. Organ responses were seen in 60% of evaluable patients with a median time to response of only two weeks after the start of treatment.

The second antibody approach, potentially applicable to all types of amyloidosis, targeted SAP. SAP binds to and is present in all amyloid deposits, which is believed to protect them from degradation by phagocytic cells and proteolytic enzymes (14). ((R)-1-(6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexa-noyl) pyrrolidine-2 carboxylic acid), a novel bis (D-proline) CPHPC, a drug that cross-links pairs of circulating SAP molecules in vivo triggering their removal by the liver, rapidly and almost completely eliminates SAP from the bloodstream. By contrast, even long term treatment with CPHPC only modestly depletes SAP from amyloid deposits. Subsequent work showed that antibodies to SAP can then target the remaining SAP present in all amyloid deposits, resulting in their rapid clearance by macrophage and complement mediated mechanisms. In an open label Phase I dose escalation study of 16 patients,

a combination of CPHPC and anti-SAP antibody has been shown by ^{123}I-SAP scintigraphy to result in swift and marked removal of liver amyloid deposits with associated improvement in liver function tests (25). This therapy is currently being tested in a phase 2 trial of patients with cardiac ATTR and cardiac AL amyloidosis.

Aims and Scope of the Thesis

The introduction of this thesis provides a broad overview of the pathogenesis and spectrum of amyloidosis. It classifies the different types of amyloidosis, through a range of techniques including key imaging modalities to identify visceral organ involvement. Systemic AL amyloidosis remains the commonest form of systemic amyloidosis and treatment is focused on suppression of the precursor fibril, with systemic chemotherapy, as well as supportive care of amyloidotic organ function.

The premise of this thesis focuses on cardiac and renal amyloidosis. Chapter 3 explores the role of proteomics in the diagnosis and typing of amyloid deposits and the aim of this study was to compares its use to the more commonly used Congo red and immunohistochemical staining of tissue, as well as specialist clinical assessment.

The second part of this thesis investigated risk stratification and outcomes of patient with combined cardiac and renal amyloidotic organ dysfunction in AL amyloidosis. Chapter 5, specifically explores outcomes in patients with systemic AL amyloidosis and advanced renal excretory impairment (eGFR <20ml/min) at baseline, previously deemed too 'high risk' for chemotherapy. The aim of this study was to assess whether prompt treatment in such patients would improve both renal and overall survival.

A key feature of systemic AL amyloidosis is its multiorgan involvement. It is a recognised cause of Type 5 cardiorenal syndrome and current consensus focuses on individual organ involvement as well as response to treatment. The aim of chapter 6 was to assess the incidence of combined cardiac and renal amyloidotic organ dysfunction at baseline, test the current renal and cardiac staging systems and identify the key biomarkers both at baseline and during follow up, that dictate the hard outcomes of death, dialysis and their composite.

Supportive care is a key part of treatment in systemic AL amyloidosis, most commonly with the use of diuretics to correct volume status and nutritional interventions to improve both treatment tolerance and quality of life. Proteinuric CKD and congestive cardiac failure can manifest with

ECV overload as well as changes in flesh weight. Clinical practice often relies on both examination and serial weights to assess patient's optimal 'dry' weight, despite sarcopenia being a recognised complication of systemic chemotherapy. The aim of chapter 7 was to explore the role of body composition in systemic AL amyloidosis, using biompedance vector analysis (BIVA), and whether it may offer insight into changes in weight as well as ECV status and the latter's correlation with the key cardiac biomarker, NT-proBNP.

The final chapter of this thesis focuses on the most challenging aspect of cardiac AL amyloidosis, early sudden cardiac death. Despite a number of studies demonstrating the presence of both tachy and brady arrhythmias in cardiac AL amyloidosis, outcomes remain poor. This chapter aimed to explore the role of intracardiac defibrillators (ICD's) and whether they provide effective treatment of arrhythmias and more importantly confer a survival benefit.

The overarching hypothesis of this work is that refinements and improvements in tissue diagnosis, biomarker based risk stratification, and supportive management of patients with renal and cardiac amyloidosis will result in preservation of organ function and improved overall survival.

Chapter Two: Materials and Methods

Declaration

I have designed the studies, carried out the data collection and the analysis of the data. I collected the data and performed the statistical analysis in my role as a clinical research fellow at the National Amyloidosis Centre, University College Medical School (Royal Free Campus). Several diagnostic methods were carried out by other individuals in the department they were as follows:

Histological and immunohistochemical analyses were performed by Janet Gilbertson.

Gene sequencing was performed by Dorota Rowczenio and Hadija Trojer.

Echocardiography was performed by Babita Pawarova, Cecil Tabadero and Sevda Ward

^{123}I-SAP scintigraphy was performed by David Hutt and Raymond Vito.

ICD monitoring was reviewed by Dr Carol Whelan, Dr Dominic Rogers and Dr Farhar Khan.

Measurement for biochemical and haematological data were performed by the Royal Free Hospital laboratory services.

Statistics advice was given by Aviva Petrie from the Biostatistics Unit at the UCL Eastman Dental Institute for chapters 3-7.

Patients

All patients in this thesis were seen at the UK National Amyloidosis Centre. An access database has been kept up to date with details of patients who have been referred to the NAC with suspected amyloidosis. All the patients included within the database have given explicit informed consent.

SAP Scintigraphy

SAP scintigraphy was performed in all patients seen at the centre with suspected amyloidosis at baseline and in those in whom it was clinically indicated at set time points during their follow up. Each subject undergoing SAP scintigraphy received approximately 200μg of SAP with 190MBq of ^{123}I, the equivalent of 3.8 mSV of radiation. Thyroid uptake was blocked by the administration of 60mg of potassium iodide immediately prior to the study and 5 further doses were given over the following three days. Anterior and posterior imaging was performed at either 6 or 24 hours after injection using an IGE-Starcam gamma-camera (IGE Medical Systems, Slough, UK). Female patients were asked to confirm that they were not pregnant prior to undergoing SAP scintigraphy.

Amyloid load was classified according to 4 criteria. Normal, small, moderate and large. 'Normal' was defined as no evidence of abnormal tracer localization. 'Small' was defined as uptake in one or more organs whilst still maintaining normal intensity in the blood pool and 'moderate' when uptake was seen in one or more organs and the blood pool was diminished. 'Large' was defined as uptake in one or more organs with no evidence of tracer in the blood pool despite adjustment of the grey scale to encompass the visceral organs involved.

Progression of amyloid by SAP scintigraphy was defined as an increment within the 4 category staging system and regression of amyloid by SAP scintigraphy was defined as a decrement within the categorical staging of amyloid load.

Cardiac Assessment

Assessment of cardiac amyloidosis is performed using a combination of clinical parameters, serum biomarkers and imaging modalities.

All patients referred to the centre with suspected cardiac amyloidosis underwent testing of serum cardiac biomarkers as well as electrocardiography and echocardiography. All new

patients where appropriate since December 2015 underwent CMR at the National Amyloidosis Centre or if appropriate had their images reviewed by Dr Marianna Fontana or Dr Dan Knight.

Functional Assessment

Functional evaluation of patients was performed using a combination of subjective and objective parameters. New York Heart Association Classification (NYHA) was used to assess for symptoms of heart failure (Table 2.1). Eastern Co-operative Group (ECOG) performance status was used (Table 2.2), particularly in patients with systemic AL amyloidosis receiving cytotoxic therapy in order to assess their tolerance to treatment. A history entailing baseline exercise tolerance is taken during the clinical consultation and for all patients able to perform it, a 6 minute walk test (6MWT) is completed with both a total number of metres walked and percentage predicted for age calculated. 6MWT is performed according to standardised criteria(111) and has been shown to be a valuable measure of functional change in patients with systemic AL amyloidosis receiving chemotherapy(112).

Table 2.1: NYHA classification

NYHA Class	**Description**
Class 1	No symptoms and no limitation in ordinary physical activity, e.g. shortness of breath when walking, climbing stairs etc.
Class 2	Mild symptoms (mild shortness of breath and/or angina) and slight limitation during ordinary activity.
Class 3	Marked limitation in activity due to symptoms, even during less-than-ordinary activity, e.g. walking short distances (20-100 m). Comfortable only at rest.
Class 4	Severe limitations. Experiences symptoms even while at rest. Mostly bedbound patients.

Table 2.2 Definition of Eastern Co-operative Group Performance Status

Grade	Summary	Description
0	Normal	No restriction to carrying out normal activities
1	With effort	Ambulatory, able to do light work. Restricted only in strenuous
2	Restricted	Self-caring and ambulatory but unable to carry out work
3	Dependent	Capable of limited self-care, confined to bed or chair for over
4	Immobile	Unable to carry out self-care, completely confined to bed or chair

Echocardiography

Echocardiography was performed in all patients with two-dimensional and M-mode settings using a GE Vivid 7 system. Parasternal long axis and apical long axis views were most commonly used. Evaluation of left ventricular wall thickness, left ventricular diastolic function, left ventricular systolic function and atrial diameter were measured 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).

Electrocardiogram

Low voltage amplitude was defined by a mean QRS amplitude in leads I, II, II, AVL and AVF of less than 0.5mV. (113)

Holter Monitoring

Twenty-four hour electrocardiographic monitoring (Holter monitoring) was performed using a portable cassette recorder with three lead placements (Spacelabs Healthcare Lifecard CF). Analysis of the 24 hour records was performed by an experienced electro

physiologist using Pathfinder Digital software V8.701 (Spacelabs). Two consultant cardiologists reviewed the reports. Referring physicians were contacted with the results of significant results and changes to management were recommended based on current clinical practice for cardiac rhythm disturbances.

Criteria for Diagnosis of Amyloid and Definition of Organ Response

The definition of organ involvement and organ response was defined according to consensus criteria in combination with SAP scintigraphy (Table 2.3). (69)

Table 2.3 Definition of Organ Involvement and Organ Response

Organ	Definition of Organ Involvement	Definition of Organ Response
Heart	Echocardiogram: Mean wall thickness >12mm and no other cardiac cause or CMR showing late gadolinium enhancement	Mean IVSd decreased by 2mm, 20% improvement in EF, improvement by 2 NYHA classes without an increase in diuretic use and no increase in wall thickness
Kidneys	24 hour non Bence Jones Proteinuria >0.5g, or uptake on SAP scintigraphy	≥ 50% reduction in proteinuria (at least 0.5g/day) without a ≥ 25% reduction in eGFR, or increase in serum creatinine.
Liver	SAP scintigraphy	50% decrease in abnormal ALP or reduced organ uptake on SAP scintigraphy
Spleen	SAP scintigraphy	Reduced organ uptake on SAP scintigraphy
Adrenal	SAP scintigraphy	Reduced organ uptake on SAP scintigraphy
Soft Tissue	Tongue hypertrophy, periorbital bruising, spontaneous bruising, pseudo hypertrophy, lymphadenopathy, carpal tunnel syndrome	Clinical assessment of improvement
Gastrointestinal Tract	Direct biopsy verification with symptoms	
Lung	Direct biopsy verification with symptoms, interstitial radiographic pattern	Radiographic evidence of improvement in pulmonary interstitial amyloid (rare)

Peripheral Neuropathy	Symmetrical sensorimotor peripheral neuropathy in the lower limbs	Clinical assessment
Autonomic Neuropathy	Impotence, diarrhoea or constipation, early satiety and/or impaired bladder emptying without other overt cause. Orthostatic hypotension (>20mmHb fall in systolic BP)	Clinical Assessment

Histology

Congo red Staining

Formalin fixed de-paraffinised tissue sections 6-8µg thick were rehydrated, and counterstained with haematoxylin under running tap water. Sections were then stained using the alkaline-alcoholic Congo-red method as previously described by Puchtler *et al*. (50) A series of ascending ethanol concentrations to xylene were used to dehydrate the sections which were then mounted in DPX mounting medium. Stained slides were then viewed in bright field and under cross polarised light. Positive controls were obtained from a known Congo-red positive block validated by laser micro dissection and mass-spectrometry based proteomic analysis which was always processed in parallel.

Immunohistochemistry

The amyloid type was then characterised by immunohistochemical staining. Formalin fixed de-paraffinised 2µm sections of amyloidotic tissue were used. Sections were washed with water and endogenous peroxidise activity was quenched by incubation in aqueous (0.3%) hydrogen peroxide (H_2O_2) for 30 minutes. They were then rinsed again in phosphate-buffered saline (PBS) containing 0.05% Tween (Calbiochem). Prior to the application of antisera, non specific tissue binding was abolished by incubation for a further 30 minutes in normal non-immune serum from the species providing the secondary antibody (Vector Part of the ImmPRESS Kit). Sections were then incubated overnight with primary antisera at 4^{o}C. They were rinsed with PBS containing 0.05% Tween (Calbiochem) and labelled with secondary antibodies. Sections were washed in PBS and bound enzyme-antibody bound complexes were then visualised using a metal-enhanced DAB (Fisher Scientific solution).

A panel of anti-human monospecific antibodies reactive with: SAA (Eurodiagnostica, Huntington UK) AL kappa, lambda, transthyretin and lysozyme (Dako Ltd,

Denmark House Ely UK), Apolipoprotein AI (Genzyme Diagnostics) and fibrinogen Aα chain (Calbiochem) were used where appropriate. Congo red overlay was used in duplicate sections. Immunohistochemically stained sections were counterstained in haematoxylin, 'blued' under running tap water and stained with Congo-red. (114)

For TTR staining, pre-treatment was performed for enhanced antigen retrieval using 10 minute incubation 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 in 0.9% sodium chloride.

Laser Dissection Mass Spectrometry

For microdissection, stained CR sections were viewed under brightfield and florescence light using the Leica LMD7 laser capture microscope. Areas positive for amyloid when viewed using fluorescence light at excitation 497 nm and emission 614 nm wavelengths were laser microdissected into micro-centrifuge caps. Proteins were extracted from each sample into 10 mM Tris/1 mM EDTA/0.002% Zwittergent buffer solution (35 μl) by heating (99 °C, 1.5 h) followed by sonication (1 h) and then digested with trypsin (25 ng/sample) overnight (~ 18 h) at 37 °C. Each digested sample was reduced with dithiothreitol (50 μg) at 99 °C for 5 min, freeze dried, reconstituted in 0.1% v/v trifluoroacetic acid in HPLC grade water (20 μl) and analysed by HPLC-MSMS, as previously described.(115, 116)

MS raw data files were queried using Mascot and assigned peptide and protein probability scores. Amyloid by LDMS was established on the basis of the presence of the 'amyloid signature proteins (ASP)' defined by presence (≥ 1 unique specific peptide) of two or more of the following proteins; apolipoprotein E (APOE), apolipoprotein A-IV (APOA4) and serum amyloid P component (SAP), as previously described.(59) The amyloid fibril protein was determined by presence of Mascot score of >80 coupled with at least 2 unique specific peptides of a known amyloid fibril protein together with absence (Mascot score < 80 or fewer than 2 unique specific peptides) of other known amyloid fibril proteins.

Immunoassays

Serum Amyloid A Protein

SAA levels were measured using latex nephelometry (BNII autoanalyser Dade, Behring Marbury, Germany). (117) The lower limit of detection is 0.7mg/L. Standardisation was based on WHO international reference standards 1987. (118)

Serum Free Immunoglobulin Light Chain Assay

Both kappa and lambda serum free immunoglobulin light chains (FLC) were measured using a latex-enhanced immunoassay (The Binding Site, Birmingham, UK) on a ehring BNII autoanalyser (Dade Behring, Marburg, Germany). (119-121) Antibodies are directed against FLC epitopes hidden within whole immunoglobulin molecules. The sensitivity of the assay is <5mg/L. Sera from 100 healthy blood donors were tested in order to determine the reference range. The mean concentrations of polyclonal free kappa and free lambda light chains were 11.38mg/L (95% CI, 7.41-16.77mg/L) and 17.36mg/L ((%% CI, 8.91-29.87mg/l) respectively. The mean kappa/lambda ratio was 0.7 (95% CI, 0.37-0.95). An abnormally high kappa or lambda light chain value or abnormal ratio in the context of preserved renal function was used as part of the assessment of an underlying clonal disorder in chapter four. In patients with renal impairment the ratio alone was used. The definitions of haematologic response are outlined in Table 2.4.

Light chains are metabolised in the kidneys. Polyclonal free light chain levels rise in renal failure(122) which makes interpretation of absolute levels of serum free light chains difficult to interpret. If the 'normal' ratio of kappa/lambda is estimated to be 1:1, the amount of monoclonal light chain can be estimated by subtracting the uninvolved light chain from the involved light chain, a method previously validated in myeloma.(123) This method has been used when calculating the light chain response in chapters five and six.

Table 2.4 Haematologic Response Criteria (69)

Clonal Response	Criteria
Complete Response	Serum and urine negative for a monoclonal protein by immunofixation, normal free light chain ratio
Partial Response	If serum paraprotein >0.5g/dL, a 50% reduction If light chain in the urine with a visible peak and >100mg/day and 50% reduction If free light chain >10mg/dL (100mg/L) and 50% reduction
Progression	From CR, any detectable monoclonal protein or abnormal free light chain ratio (doubling of light chain) From PR or stable response, 50% increase in serum paraprotein to >0.5g/dL or 50% increase in urine paraprotein to >200mg/day Free light chain increase of 50% to >10mg/dL (100mg/L)
Stable	No CR, no PR, no progression

Gene Sequencing

Genotyping was performed in patients with suspected hereditary amyloidosis where appropriate. Whole blood taken in an EDTA tube was frozen and stored for gene sequencing as required. Genomic DNA was isolated by a rapid method. 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. Polymerase chain reaction (PCR) using 'Ready-To-Go' tubes (GE Healthcare) were used to amplify the coding regions for the following genes: transthyretin (exons 2,3 and 4), apolipoprotein AI (exons 3 and 4) and fibrinogen A α-chain (exon 5). HotStar Taq DNA Polymerase kit (Qiagen) was used for the lysozyme gene (exon 2). The primers used as part of the PCR process are outlined in Table 2.5.

Table 2.5 Primers Used in the PCR Process for Genotyping Hereditary Amyloidosis

Gene	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'-
Apolipoprotein (4)	5'-CACTGCACCTCCGCGGACA-3'	5'-

Fibrinogen (5)	5’-AGCTCTGTATCTGGTAGTACT-3’	5’- ATCGGCTTCACTTCCGGC-3’
Lysozyme (2)	5’-GTTATATTGTTCGTTGGTGT-3’	5’- CATTTGTATTGAGTCTCAATTC-3’

Statistical Analysis

Statistical analyses were performed using Graph Pad Prism (Version 5.03),

IBM SPSS Statistics 23 (SPSS Inc, Chicago III) and Stata version 14

(Statacorp).

Individual statistical methods are discussed separately in each results chapter.

Results

Chapter Three: The complementary role of histology and proteomics for diagnosis and typing of systemic amyloidosis

Introduction

The amyloidoses are a group of rare diseases caused by extracellular accumulation of amyloid, a fibrillar material derived from a variety of precursor proteins that can aggregate in a highly abnormal cross B sheet conformation.(1, 2) Amyloid deposits progressively disrupt tissue structure and function,(3) the various clinical syndromes being classified according to the respective fibril precursor protein, of which more than 30 are known.(124) Amyloid is identified by the pathognomonic finding of apple-green dichroism when affected tissue sections are stained with Congo red dye and visualized under polarized light microscopy. Under electron microscopy, amyloid fibrils have a characteristic appearance of rigid non-branching fibrils with a diameter of ~10 nm.(5)

Systemic amyloidosis is highly heterogeneous not only with respect to amyloid fibril protein type but also to the range of organ involvement and broader clinical phenotype. Therapy is aimed at reducing the supply of the respective amyloid fibril precursor protein, and there is therefore a critical need to definitively determine the amyloid fibril protein type in every patient.(125) For example, chemotherapy, which can be very toxic, is potentially beneficial only in AL amyloidosis, which can be particularly challenging to confirm.(51)

Congo red (CR) histology followed by immunohistochemical (IHC) staining of biopsy samples is the classical and widely available method for identifying amyloid and determining the fibril type. Whilst this is the historical gold standard for diagnosis and typing of amyloid,

many different staining methods and antisera are used in different labs, and substantial operator experience is required for best results. The highly idiosyncratic nature of monoclonal immunoglobulin light chain (AL) presents particular challenges. Consequently, the sensitivity and specificity of IHC varies with type of amyloid, local methods and experience.(126) IHC studies of thousands of samples in our own centre have failed to confirm amyloid type beyond doubt in up to 30% of cases, mostly when AL amyloidosis is probable on clinical grounds. Immunofluorescence (IF) is a more reliable technique for the typing of AL amyloid deposits than IHC,(127) although it requires frozen amyloidotic tissue. Immunoelectron microscropy (IEM) is a technique that combines IHC and electron microscopy and allows for the correct characterisation of the amyloid protein in virtually all cases but IEM is not widely available and is currently performed in only a select number of specialist centres.(128)

Laser dissection and tandem mass spectrometry (LDMS) is an alternative, reportedly accurate tool for identification and typing of amyloid deposits which has gained popularity. Importantly, it can be performed using tiny quantities of formalin fixed amyloidotic tissue. LDMS was first validated by the Mayo group in 2009, in 102 predominantly endomyocardial biopsy specimens; LDMS was 98-100% sensitive and specific in comparison to clinicopathologic criteria for identification and typing of amyloid.(59)

We sought here to compare and evaluate the roles of CR/IHC and LDMS for identifying and typing amyloid in a diverse population of amyloidosis patients referred to our single national centre.

Methods

Samples

During the calendar year 2017, 1864 formalin–fixed paraffin wax-embedded (FFPE) biopsies from various tissues of patients suspected to have amyloid were studied in the UK National Amyloidosis Centre (NAC). They were received from many local hospitals across the UK and overseas. Tissue fixation and processing into paraffin blocks had been performed according to

the referring hospitals' protocols and was therefore not standardized. Details on the duration of fixation and processing were unavailable.

Sections from all FFPE biopsy samples were cut and stained with Congo red and a panel of anti-fibril protein antibodies as described below. Amyloid, identified by pathognomonic green birefringence of Congo red stained tissue sections viewed under crossed polarized light, was present in 1109/1864 (59%) biopsy samples (CR+ve); 755 (41%) were negative for presence of amyloid (CR-ve). The amyloid fibril protein was determined by IHC in 789/1109 (71%) CR+ve samples, but could not be characterised definitively in the remaining 320 CR+ve samples.

For purposes of this study, we processed a total of 700 samples for LDMS comprising all 320 CR+ve samples in which IHC was non-diagnostic of the amyloid fibril protein, 320 randomly selected CR+ve biopsy samples in which the amyloid type was definitively determined by IHC, and 60 randomly selected CR-ve biopsy samples.

All patients were managed in accordance with the declaration of Helsinki and institutional review board approval from the Royal Free Hospital Ethics committee was obtained for this study (REC/06/Q0501/42).

Congo red (CR) and immunohistochemical (IHC) staining at NAC

Twenty-two serial sections were cut from each FFPE block for CR and IHC staining. Sections of 2 µm thick were used for IHC and sections of 6 µm thick were used for CR staining and IHC/CR overlay.(114) Congo red staining was by the method of Puchtler et al and amyloid was identified by presence of apple green birefringence when viewed under crossed polarised light.(50) Immunohistochemical staining was with a panel of antibodies against known amyloid fibril proteins using the Shandon Sequenza™, namely, kappa and lambda immunoglobulin light chains, transthyretin, amyloid A protein, fibrinogen, LECT2, apolipoprotein A-I, and lysozyme, as previously described.(129) Antigen retrieval was not performed with the exception of transthyretin staining, which uses oxidation steps 1% aqueous sodium periodate (10min) and 0.1% sodium borohydride (10min) followed by 6 M guanidine (4h). Sections were blocked for

endogenous peroxidases and also blocked with normal serum. Sections were incubated overnight at 4°C with the primary antibodies, rinsed in PBS. The antibodies were detected with the appropriate IMMPRESS™ (Vector Laboratories) polymer detection kit and labelled using metal enhanced DAB chromagen (Thermo Scientific).

Presence of amyloid by CR staining (CR+ve) was defined by presence of pathognomonic apple green birefringence when viewed under crossed polarised light. The amyloid fibril protein was established on the basis of unique and specific staining of the amyloid with an antibody from the amyloid fibril protein panel. Interpretation was carried out without any clincial information by two independent experienced assessors using a Leica DM4000 with and without crossed polarising filters.

Laser capture microdissection and proteomic mass spectrometry analysis

For microdissection, stained CR sections were viewed under brightfield and florescence light using the Leica LMD7 laser capture microscope. Areas positive for amyloid when viewed using fluorescence light at excitation 497 nm and emission 614 nm wavelengths were laser microdissected into micro-centrifuge caps. In tissue samples where Congo red staining was equivocal or scanty, usually as a result of presence of areas of amorphous eosinophilic material under brightfield light with seemingly white birefringence but bright fluorescence, such areas were captured by the same method. In renal tissues in which the differential diagnosis included amyloid or non-amyloid monoclonal gammopathy of renal significance (MGRS) and in which presence of amyloid was not confirmed by CR staining, glomeruli were captured by the same method. Proteins were extracted from each sample into 10 mM Tris/1 mM EDTA/0.002% Zwittergent buffer solution (35 µl) by heating (99 °C, 1.5 h) followed by sonication (1 h) and then digested with trypsin (25 ng/sample) overnight (~ 18 h) at 37 °C. Each digested sample was reduced with dithiothreitol (50 µg) at 99 °C for 5 min, freeze dried, reconstituted in 0.1% v/v trifluoroacetic acid in HPLC grade water (20 µl) and analysed by HPLC-MSMS, as previously described.(115, 116)

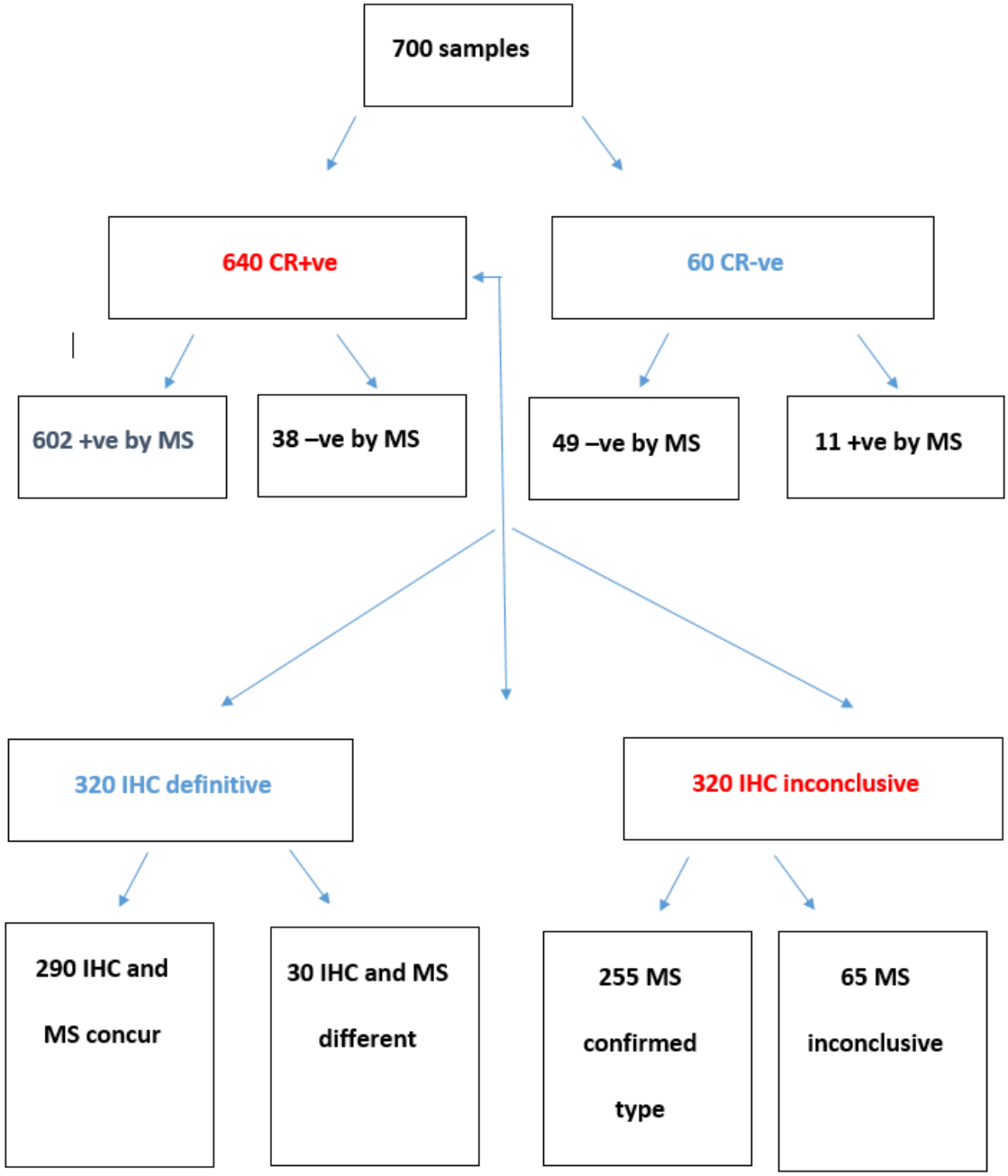
MS raw data files were queried using Mascot and assigned peptide and protein probability scores. Amyloid by LDMS was established on the basis of the presence of the 'amyloid signature proteins (ASP)' defined by presence (≥ 1 unique specific peptide) of two or more of the following proteins; apolipoprotein E (APOE), apolipoprotein A-IV (APOA4) and serum amyloid P component (SAP), as previously described.(59) The amyloid fibril protein was determined by presence of Mascot score of >80 coupled with at least 2 unique specific peptides of a known amyloid fibril protein together with absence (Mascot score < 80 or fewer than 2 unique specific peptides) of other known amyloid fibril proteins.

Analyses

Results of CR and IHC were compared with results of LDMS in a total of 700 samples (Figure 3.1). Specifically, they were compared with respect to identification of amyloid and identification of the amyloid fibril protein (amyloid type).

Figure 3.1

Flowchart of results of 700 samples processed for CR with IHC and LDMS.



Results

The 700 samples (640 CR+ve and 60 CR-ve) were from a total of 50 different tissue types with renal (n=188), cardiac (n=126) and bone marrow trephine (n=44) comprising over 50% of all samples processed.

Diagnosis of amyloid

Six-hundred and two of the 640 (94%) CR+ve samples were ASP+ve by MS and 38 (6%) were ASP-ve. Fifteen of 38 (39%) CR+ve/ASP-ve samples were classified by MS as 'insufficient for analysis' due to scanty tissue (n=14) or scanty amyloid (n=1) with a total of < 5 proteins identified within the sample. In the remaining 23/38 (61%) CR+ve/ASP-ve samples there was adequate tissue for analysis. The details of these 23 tissues samples (obtained from 20 patients) are listed in Table 3.1 which also includes the findings of a comprehensive clinical, biochemical and imaging review that was conducted in 15/20 individuals. In each of the 15 cases, an unequivocal diagnosis of amyloidosis was established on the basis of combined clinical, biochemical, echocardiographic, cardiac magnetic resonance imaging (CMR), and scintigraphic analyses.(130) The final diagnosis was localised AL amyloidosis in 4 cases, ATTR amyloidosis in 4 cases, systemic AL amyloidosis in 3 cases, Leukocyte Chemotactic Factor-2 (ALect2) amyloidosis in 2 cases, beta 2 microglobulin (β2M) amyloidosis in 1 case, and the final patient was a 59 year old male with carpal tunnel amyloid deposits in the absence of any critical amyloidotic organ involvement or dysfunction. The remaining 5/20 patients were not seen at our centre, and their biopsy sample had been sent to NAC solely for histological analysis.

Forty-nine of the 60 (82%) CR-ve samples were ASP-ve by MS and 11/60 (18%) were ASP+ve (Table 3.2). There were no CR-ve/ASP+ve cardiac, gastrointestinal, fat or bone marrow trephine biopsy samples (Table 3.3). However, there were 7 renal biopsy specimens, all glomerular, from a total of 188 (4%) renal biopsies which were CR-ve/ASP+ve. In 4/7 such cases, a diagnosis of fibrillary glomerulonephritis (GN) was established on the basis of the clinical and electron microscopy findings coupled with presence of DNAJB9 protein by MS.(131) One of the remaining 3 patients with a CR-ve/ASP+ve glomerular sample was

eventually diagnosed with fibronectin glomerulopathy, and the diagnosis in the remaining 2 cases was not established at the time of writing (Table 3.2).

Table 3.1. Samples diagnostic of amyloid by Congo red staining but with no amyloid by MS (CR+ve/ASP-ve)

Sample / Patient No	Age & Sex	Tissue	Clinical / Histology review	Final Diagnosis*	Organ Involvement	SAP scintigraphy	DPD scan
1/1	62 F	Lymph node	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid	Not done
2/2	87 M	Fat aspirate	Clinical	ATTR amyloidosis	Cardiac	No visceral amyloid	Grade 2
3/3	65 M	Prostate	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid	Not done
4/4	67 M	Renal	Clinical	Systemic AL amyloidosis	Renal	Isolated renal amyloid	Not done
5/5	84 M	Carpal tunnel	Clinical	Beta 2 microglobulin amyloidosis	No vital organ involvement	No visceral amyloid	Not done
6/6	68 F	Renal	Clinical	ALECT2 amyloidosis	Renal	Amyloid in spleen and kidneys	Not done
7/7	67 F	BMT	Histology	Uncertain	Not done	Not done	Not done
8/8	55 M	Muscle	Histology	Uncertain	Not done	Not done	Not done
9/9	84 M	Cardiac	Clinical	ATTR amyloidosis	Cardiac	No visceral amyloid	Grade 2
10/10	68 M	Tonsil	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid	Not done
11/11	82 M	Tongue	Clinical	ATTR amyloidosis	Cardiac	No visceral amyloid	Grade 2
12/12	54 M	Brain	Histology	Uncertain	Not done	Not done	Not done
13/13	72 M	BMT	Clinical	Systemic AL amyloidosis	Cardiac and soft tissue	No visceral amyloid	Not done
14/14	74 M	Renal (glomeruli)	Clinical	Renal amyloid – uncertain type	Renal	Isolated renal amyloid	Not done
15/14		Renal (medulla)	*As above*	*As above*	*As above*	*As above*	*As above*
16/15	34 F	Mucosa	Histology	Uncertain	Not done	Not done	Not done
17/16	63 M	Skin	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid	Not done
18/17	68 M	Renal (interstitium)	Histology	ALECT2 amyloidosis	Not done	Not done	Not done
19/18	59 M	Carpal tunnel	Clinical	Amyloid – uncertain type	No vital organ involvement	No visceral amyloid	Grade 0
20/19	92 M	Prostate	Clinical	ATTR amyloidosis	Cardiac	No visceral amyloid	Grade 2
21/19		Fat aspirate	*As above*	*As above*	*As above*	*As above*	*As above*
22/19		Fat aspirate #2	*As above*	*As above*	*As above*	*As above*	*As above*
23/20	61 F	BMT	Clinical	Systemic AL amyloidosis	Cardiac and soft tissue	Amyloid in liver and spleen	Not done

*The final diagnosis in patients who underwent clinical review was established on the basis of a comprehensive clinical assessment, biochemical investigations including serum and urine immunofixation, serum free light chains and cardiac biomarkers and specialist imaging including SAP scintigraphy, DPD scintigraphy, echocardiography and cardiac magnetic resonance imaging.

Table 3.2. Samples with no amyloid by Congo red staining but with presence of the 'amyloid signature' by MS (CR-ve/ASP+ve)

Sample / Patient No	Age & Sex	Tissue	Clinical / Histology review	Final Diagnosis*	Organ Involvement	SAP scintigraphy
1/1	58 F	Soft tissue	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid
2/2	52 M	Soft tissue	Clinical	Systemic AA amyloidosis	Renal	Amyloid in spleen and kidneys
3/3	72 F	Skin	Clinical	Systemic AL amyloidosis	Cardiac and soft tissue	No visceral amyloid
4/4	66 M	Prostate	Clinical	Localised AL amyloidosis	No vital organ involvement	No visceral amyloid
5/5	66 F	Renal	Histology	Fibrillary GN	Not done	Not done
6/6	76 M	Renal	Histology	Fibronectin glomerulopathy	Not done	Not done
7/7	39 M	Renal	Clinical	Fibrillary GN	Renal	No visceral amyloid
8/8	52 M	Renal	Clinical	Fibrillary GN	Renal	No visceral amyloid
9/9	46 M	Renal	Histology	Uncertain	Not done	Not done
10/10	19 M	Renal	Histology	Uncertain	Not done	Not done
11/11	61 F	Renal	Clinical	Fibrillary GN	Renal	No visceral amyloid

*The final diagnosis in patients who underwent clinical review was established on the basis of a comprehensive clinical assessment, biochemical investigations including serum and urine immunofixation, serum free light chains and cardiac biomarkers and specialist imaging including SAP scintigraphy, DPD scintigraphy, echocardiography and cardiac magnetic resonance imaging.

Table 3.3. Comparison of Congo red histology and LDMS findings in commoner tissue types

Tissue Type	Number of samples (N)	CR +ve (N)	CR –ve (N)	CR -ve/ASP + ve (N)	CR +ve/ASP –ve (N; %*)	Definitive typing of amyloid by MS (N; %*)	MS non-diagnostic of amyloid type (N, %*)	Inadequate Sample (N; %*)
Cardiac	126	117	9	0	7 (6)	103 (88)	4 (3)	3 (3)
Renal	188	170	18	7	12 (7)	139 (82)	18 (11)	1 (1)
Gastrointestinal	40	40	0	0	0 (0)	38 (95)	2 (5)	0 (0)
Fat aspirate	25	23	2	0	4 (17)	18 (78)	1 (4)	0 (0)
Bone marrow trephine	44	40	4	0	4 (10)	30 (75)	4 (10)	2 (5)

*Percentages are calculated as the percentage of all CR +ve samples (rather than percentage of all CR +ve/ASP +ve samples)

Typing of amyloid

Of the 320 samples in which the amyloid fibril protein was determined by IHC, the amyloid type by MS concurred in 290/320 (91%), whereas in 30 samples there was a discrepancy between the amyloid type identified by IHC and the MS results. This discrepancy was due to absence of ASP (i.e., CR+ve/ASP-ve) by MS in 13/30 (43%) cases and in the remaining 17 samples, the MS was either non-diagnostic of amyloid type (n=10) or discordant (n=7) (Table 3.4). Of those 7 cases in which the amyloid fibril protein by IHC and MS were discordant, 4 were AL lambda sub-type by IHC but AL kappa sub-type by MS. Two of the 4 relevant patients had a circulating monoclonal protein, in both cases, of lambda isotype. The amyloid fibril protein was discordant in a further 3/7 samples; 2 were from the same patient (renal and testicular biopsy) in which IHC showed AA amyloid and MS showed AApoAI amyloid and in whom a novel *APOA1* mutation was discovered, and the remaining sample was a bladder biopsy in which AL (lambda sub-type) amyloid was identified by IHC and MS revealed ATTR amyloid. This last patient did not undergo clinical evaluation at our centre.

Of the 320 CR+ve samples in which the amyloid type was not diagnosed by IHC, amyloid was identified and typed by MS in 255 (80%) cases but was not definitively typed in the remaining 65 cases. In the 255 samples which were inconclusive by IHC but definitely typed by MS, the latter correlated with extensive clinical assessment in 252/255 (99%) cases. In the remaining 3 cases, tissue was received for a histology review alone such that limited clinical details were available. MS established a diagnosis of; AL kappa in 135 cases, AL lambda in 55 cases, transthyretin (ATTR) in 26 cases, fibrinogen α chain (AFib) in 8 cases, localised semenogelin amyloid deposits in 6 cases, immunoglobulin heavy chain (AH) in 6 cases, hereditary apolipoprotein AI (AApoAI) in 6 cases, apolipoprotein C2 (AApoC2) in 4 cases, apolipoprotein A-IV (AApoAIV) in 4 cases, galectin 7 (cutaneous) amyloid deposits in 3 cases, insulin derived (AIns) amyloid deposits in one case and isolated atrial amyloidosis (IAA) in the final case.

Of the 65 cases in which the amyloid type could not be determined by MS, 12 samples were ‘insufficient for analysis’ by MS, 25 samples were ASP-ve, and 7 contained no known

amyloid fibril protein. Twenty one samples contained more than one known amyloid fibril protein each with a similar Mascot and specific peptide score including 7 cases with both TTR and a light chain (either kappa or lambda), and 5 cases with both kappa and lambda light chains.

Table 3.4. Samples in which MS was either non-diagnostic of amyloid type (n=10) or discordant (n=7) with results of IHC

Sample / Patient No	Age / Sex	Clinical / Histology review	Tissue	Final Diagnosis*	Organ Involvement	Systemic clone (N/ κ / λ)	IHC	MS	SAP scintigraphy
1/1	78 M	Clinical	Laryngeal	Localised AL amyloidosis	No vital organ involvement	No	AL (λ)	AL (κ)	No visceral amyloid
2/2	65 F	Clinical	Lymph node	Localised AL amyloidosis	No vital organ involvement	Lambda	AL (λ)	AL (κ)	No visceral amyloid
3/3	84 F	Clinical	Renal	Systemic AL amyloidosis	Renal	Lambda	AL (λ)	Uncertain	Isolated renal amyloid
4/4	83 M	Histology	Cardiac	ATTR amyloidosis	Cardiac	Not known	ATTR	Uncertain	Not done
5/5	25 F	Clinical	Laryngeal	Localised AL amyloidosis	No vital organ involvement	No	AL (λ)	Uncertain	No visceral amyloid
6/6	58 M	Histology	Renal	Amyloid – uncertain type	Renal	Not known	AL (λ)	Uncertain	Not done
7/7	64 F	Clinical	Breast	Localised AL amylodosis	No vital organ involvement	No	AL (λ)	AL (κ)	No visceral amyloid
8/8	79 M	Clinical	Renal	Systemic AL amyloidosis	Renal and Liver	Lambda	AL (λ)	Uncertain	Amyloid in liver and kidneys
9/9	78 M	Clinical	Cardiac	ATTR amyloidosis	Cardiac	Kappa	ATTR	Uncertain	No visceral amyloid
10/10	66 M	Clinical	Cardiac	Systemic AL amyloidosis	Renal and autonomic nerve	Lambda	AL (λ)	Uncertain	Amyloid in liver, spleen and kidneys
11/11	77 M	Histology	Bladder	Amyloid – uncertain type	Bladder	Not known	AL (λ)	TTR	Not done
12/12	67 F	Clinical	Lymph node	Insulin amyloid	No vital organ involvement	No	AIns	Uncertain	No visceral amyloid
13/13	46 M	Histology	Testes	Amyloid – uncertain type	Renal and testicular	Not known	AA	AApoAI	Not done
14/13		*As above*	Renal	*As above*	*As above*	*As above*	AA	AApoAI	*As above*
15/14	74 M	Clinical	Renal	Systemic AL amyloidosis	Renal	Lambda	AL (λ)	AL (κ)	Amyloid in spleen and kidneys
16/15	82 F	Histology	Soft tissue	Insulin amyloid	Soft tissue	Not known	AIns	Uncertain	Not done
17/16	47 F	Clinical	Laryngeal	Localised AL amyloidosis	No vital organ involvement	No	AL (λ)	Uncertain	No visceral amyloid

*The final diagnosis in patients who underwent clinical review was established on the basis of a comprehensive clinical assessment, biochemical investigations including serum and urine immunofixation, serum free light chains and cardiac biomarkers and specialist imaging including SAP scintigraphy, DPD scintigraphy, echocardiography and cardiac magnetic resonance imaging.

Discussion

CR staining and IHC remains the most commonly used method for the diagnosis and typing of amyloid worldwide, although some specialist centres have recently moved towards the use of LDMS alone.(132) Here we report for the first time a single centre direct comparison of the methods, supported where necessary by correlation with specialist clinical and imaging assessments, which highlights the advantages and limitations of each method and argues strongly in favour of their use as complementary techniques for diagnosis and typing of amyloid.

Our study showed a high concordance between CR staining and LDMS for the identification of amyloid with 94% of CR+ve samples showing amyloid by LDMS and 82% CR-ve samples not showing amyloid by LDMS. Nonetheless, LDMS in our hands failed to identify amyloid in a small proportion of cases, even when an adequate quantity of amyloidotic tissue was captured, and the reasons for this remain unclear. It is also worth highlighting that 7 of 11 CR-ve/ASP+ve were glomerular and that the final diagnosis in 5 of these 7 glomerular samples was either fibrillary GN or fibronectin glomerulopathy. In 2015 the Mayo group reported LDMS in a small cohort of MGRS cases which included 7 cases of renal amyloidosis and 8 cases of fibrillary GN. LDMS revealed the presence of 2 or more ASP (SAP and APOE) in all amyloidotic samples and presence of only APOE in fibrillary GN.(133) However, here we show that fibrillary GN and other very rare MGRS lesions, can occasionally cause 'false positives' for amyloid by LDMS. In the case of fibrillary GN however, the presence of glomerular DNAJB9 by MS should alert one to this potential risk.(131) Importantly, there were no CR-ve/ASP+ve cardiac, gastrointestinal, fat or bone marrow trephine samples such that the finding of ASP, in the absence of CR staining in these tissues, should be considered definitively diagnostic of amyloid.

With respect to typing of amyloid, MS was superior to IHC. The amyloid type was established by MS in 255 (80%) of the 320 cases that were not typed by IHC and in a total of 545 of 640 (85%) CR+ve samples. A sub-analysis according to tissue type showed that the amyloid type was definitively determined by MS in 95% of gastrointestinal, 88% of endomyocardial, 82% of renal, 78% of fat and 75% of bone marrow trephine CR+ve samples

(Table 3.3). However, despite a very high concordance between the amyloid type established by MS and that established by IHC, there were occasional discordant results (n=7; 2%) of which a proportion appeared to be more consistent with the IHC finding than that by MS, based on clinical and biochemical parameters. In addition, a number of CR+ve samples that were definitively typed by IHC did not contain ASP and the amyloid type could not therefore be determined by MS. Further, a small number of cases were not typed by MS due to presence of more than one amyloid fibril protein, each with a similar Mascot and specific peptide score.(62) It is noteworthy that these cases frequently contained a combination of immunoglobulins and transthyretin, both of which are abundant plasma proteins and may therefore be related to fixation and processing factors over which specialist referral centers, such as ours, have no control.

Whilst LDMS may successfully determine the amyloid fibril protein from tiny quantities of amyloidotic tissue, there are important factors to consider as to whether a sample is sufficient for analysis, including the distribution of amyloid deposits within the specimen. The challenges of dissecting 'chicken-wire' type amyloid deposits has been noted previously,(134) and may result in abundance of other proteins within the microdissected sample. In addition, the limitations of amyloid typing by MS on fat samples containing 'scanty' amyloid deposits has previously been highlighted.(135) Further work is needed to avoid the small proportion of samples that cannot be typed by MS. To this end, recent work from our center has looked at the role of tissue decellularization in samples in which more than one potentially amyloidogenic protein is identified. In a small case series, pre-treatment of tissue with decellularization enhanced the specificity of the identification of the correct amyloid fibril protein in samples in which more than one protein was detected.(62)

In 2015, we undertook a smaller collaborative study of a similar nature in which 142 biopsy samples were stained with CR and IHC at the NAC and processed for LDMS at the Mayo Clinic, USA. The results of this earlier study were remarkably similar to our findings here; there was a very high degree of concordance between the two techniques and LDMS enabled typing of amyloid in 74% of 34 cases that could not be typed by IHC compared to 80% here.

Furthermore, the reasons for the failure of MS to determine the amyloid type in the earlier study were listed as insufficient amyloid, insufficient tissue and technical failure,(136) almost identical to our findings here.

Our study's main limitation is that it is a retrospective analysis of biopsies from a single center. There is, by definition, a selection bias based on the fact that all patients have been suspected of having amyloidosis or MGRS by the referring histopathologist or clinician in order to reach us in the first place. Secondly, due to the fact that fixation and processing of tissue occurred in the local referring center, this was not controlled for and may have contributed to some of the failures of both IHC and LDMS;(116) as a consequence however, our study does reflect 'real-world' clinical practice.

In conclusion we show here that presence of ASP on MS is a sensitive and specific method for the identification of amyloid deposits but highlight the fact that, particularly in glomerular samples from patients with other deposition diseases such as fibrillary GN, the presence of ASP may not be due to amyloid. The addition of LDMS to CR histology and IHC markedly increased the proportion of amyloidotic samples in which the amyloid type was definitively determined to between 74% and 95%, depending on the biopsy tissue. Nonetheless, challenges remain; namely the occasional detection of more than one potential amyloid fibril protein by LDMS and the laser capture and identification of scanty material. Whilst LDMS remains a powerful tool for the diagnosis and typing of amyloid, our experience suggests that it should be used in conjunction with comprehensive clinical and histological assessment to ensure optimal patient care.

Chapter Four: Diagnosis, pathogenesis and outcome in Leucocyte chemotactic factor 2 (ALECT2) amyloidosis

Introduction

Renal amyloidosis results from the pathological deposition of amyloid protein fibrils in glomeruli and/or renal parenchyma. The diagnosis of amyloid is based on light microscopy demonstrating characteristic green birefringence in affected tissues that have been stained with Congo red and viewed under crossed polarized light. More than 30 different human proteins can form amyloid *in vivo,* which on electron microscopy appears as randomly orientated non-branching fibrils of 7-12 nm width. The commonest form of amyloid diagnosed in kidney biopsies is AL type, in which the fibrils are derived from monoclonal immunoglobulin light chain. Some 13 other proteins that can deposit as amyloid in the kidneys include serum amyloid A protein (SAA), apolipoproteins A-I, A-II and C-III, fibrinogen, lysozyme, gelsolin and transthyretin.(4)

Leucocyte chemotactic factor 2 (ALECT2) amyloidosis was first described in 2008 in a 61-year-old woman who had presented seven years earlier with nephrotic syndrome and glomerular amyloid deposits and later underwent nephrectomy for clear cell renal carcinoma.(137) Murphy *et al* subsequently described a series of ten adults with ALECT2 amyloid who presented with varying degrees of renal impairment, proteinuria and extensive interstitial and mesangial amyloid deposits. Interestingly, all affected individuals were homozygous for the G allele, a polymorphism which results in a substitution of isoleucine with valine at position 40 in the mature LECT2 protein.(138) Two recent retrospective studies, one from the Mayo Clinic and the other from Nephropath which included 72 and 40 patients respectively have established the clinical and pathological characteristics and outcomes in renal ALECT2 amyloidosis in the U.S.(139-141) A retrospective U.S. renal biopsy series of 285 samples identified ALECT2 amyloid as the third most common type of renal amyloid at 2.5%.(142) In a retrospective study of 130 cases of hepatic amyloid, the Mayo Clinic investigators

identified ALECT2 amyloid in 25% of cases making it the second commonest form of hepatic amyloid.(143) They identified unique pathological and clinical features, including the fact that the diagnosis of amyloid was often incidental, starkly contrasting with hepatic AL amyloid in which hepatomegaly and liver dysfunction are typical.

There have been few studies worldwide and none from Europe exploring long term follow up and outcome in ALECT2 amyloidosis. Here we report the prevalence, clinical presentation, diagnostic findings and long term outcome among all 24 patients who were diagnosed with ALECT2 amyloidosis at the UK National Amyloidosis Centre over a 21 year period.

Methods

Patients and Outcomes

We included in this study all patients with ALECT2 amyloidosis who attended the UK National Amyloidosis Centre over a period of greater than 20 years (1994 to 2015). Nine of 24 patients were identified to have ALECT2 amyloidosis retrospectively and the remainder were diagnosed with ALECT amyloidosis at the time of diagnosis of amyloid. Patients attended the NAC for their initial diagnostic evaluation and were followed up at regular (usually 12 monthly) intervals. Investigations undertaken at each visit to the centre included detailed blood and urine biochemistry, electrocardiography, echocardiography and monitoring of visceral organ involvement as well as whole body amyloid load by ^{123}I-SAP scintigraphy. Additional investigations were undertaken when clinically indicated. 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.

Histology and Immunohistochemistry

Renal (n=20), hepatic (n=4) and rectal (n=1) biopsies were processed, as previously described (one patient had both liver and renal biopsies). Briefly, serial sections were cut from each formalin fixed

paraffin embedded block (FFPE). Amyloid was diagnosed on Congo red staining by apple green birefringence when viewed under cross polarized light microscopy. Paraffin embedded sections of 2 µm thickness were used for IHC, and sections of 6 µm thickness were used for Congo red and IHC/Congo red overlay, as previously decribed.(114) Immunohistochemical staining of the amyloid deposits was performed using an extensive panel of monospecific antibodies reactive with serum amyloid A protein (SAA) (Euro Diagnostica AB), kappa and lambda immunoglobulin light chains (Dako), apolipoprotein A-I (Genzyme), fibrinogen Aα-chain (Calbiochem®), transthyretin (Dako), and LECT2 (R & D Systems). The goat anti-human LECT2 antibody (cat no AF722), was used at 1:600 dilution. Staining specificity was determined by prior absorption of the antiserum with purified human protein, in these cases with human LECT2, which resulted in complete abrogation of staining. IHC was performed on the Sequenza™ (Fisher Scientific, UK) platform using Impress™ (Vector laboratories UK) detection kit following the standard method, with the use of a metal enhanced DAB Substrate kit (Thermo Scientific, UK) for visualizing the immuno compound. All slides were viewed on a DM4000 (Leica Microsystems, UK) and were interpreted independently by two experienced operators.

Proteomic analysis of amyloidotic tissue

Laser micro dissection (LMD) followed by liquid chromatography and tandem mass spectrometry (MS) (LC-MS/MS) was performed on Congo red-positive glomeruli or renal tubules as previously described by Dogan and colleagues.(115) For comparison, purified human LECT2 protein was trypsinized and analyzed by LC-MS/MS on a Velos orbitrap mass spectrometer. MS data files were analyzed using Mascot(144) to search the SwissProt database (SIB Bioinformatics Resource, Bethesda, MD). Searches were conducted based on trypsin as the digestion enzyme and oxidation of methionine set as a variable modification; mass tolerances were 10 ppm for precursor ions and 0.60 Da for fragment ions.

Genetic Analysis

Genomic DNA was extracted from whole blood treated with EDTA as previously described.(145)

Coding regions of the *LECT2* gene (NCBI Ref NC_000005.10) were amplified by polymerase-chain-reaction assay (PCR) and analyzed by automated sequencing as previously described.(137) 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). The electropherograms of the *LECT2* gene were analysed on the ABI 3130xl Genetic Analyser using Sequencing Analysis Software version 5.4.

^{123}I-Labelled SAP Scintigraphy

All patients underwent whole body anterior and posterior scintigraphic imaging 24 h after administration of ^{123}I-labelled serum amyloid P component (SAP) using a GE Infinia Hawkeye gamma camera (GE Healthcare, Iowa, MN), as previously described.(65) Labelled SAP studies were interpreted by a panel of physicians with experience of over 29 000 SAP scans.

Statistical Methods

Patient survival, time from diagnosis of amyloidosis to end-stage renal disease defined as commencement of renal replacement therapy (renal survival) and survival from commencement of renal replacement therapy were estimated by Kaplan-Meier analyses. Censor date was 1st December 2015. Rate of decline of renal function was analyzed in those patients who were not requiring renal replacement therapy at the time of diagnosis, and was expressed in ml/min/yr.

Results

Patients

Twenty-four patients were confirmed to have ALECT2 amyloidosis at the UK National Amyloidosis Centre (NAC), comprising 0.36% of 6739 patients with amyloidosis and 1.3% of 1853 patients with biopsy proven renal amyloid seen at the centre between 1994 and 2015. Of these 24 cases, 9 were diagnosed retrospectively after the discovery of ALECT2 amyloidosis in 2008 having previously

been classified as amyloid with no immunospecific staining. Twenty-two patients had a renal presentation with proteinuria and/or impaired renal excretory function, including 20 who had been referred to the NAC on the basis of amyloid detected in a kidney biopsy. Four patients had amyloid detected on liver histology (including one with established amyloid deposits in the kidneys), performed in 3 of the 4 cases to investigate chronic viral hepatitis infection and in the last case as work up for peritoneal pseudomyxoma. The diagnosis of ALECT2 amyloidosis was therefore established on renal histology in 20 cases, liver histology in 3 cases and on rectal histology in the remaining case (Table 4.1).

Table 4.1. Patient demographics in ALECT2 amyloidosis

No	Age at diagnosis Sex	Ethnic Origin	Histology	Hepatitis B/C	Serum creatinine (µmol/L)	eGFR (ml/min)	Proteinuria (g/24hr)	SAP scintigraphy (Liver/Spleen/ Kidneys/Adrenals)
1	61 M	Egyptian	Renal and hepatic	C	201	30	0.17	L/S/K
2	62 M	Egyptian	Renal	-ve	245	24	0.10	L/S
3	58 F	Egyptian	Renal	-ve	116	44	0.47	L/S/K/A
4	62 M	Egyptian	Renal	-ve	219	27	0.48	K/A
5	57 M	Egyptian	Hepatic	-ve	94	76	1.11	L/S/K/A
6	66 M	Indian	Renal	-ve	379	15	0.90	L/S
7	37 M	Indian	Renal	-ve	74	110	0.10	Normal
8	74 M	Indian	Renal	-ve	181	33	0.10	S/K
9	63 M	Indian	Renal	-ve	156	40	2.52	S/K/A
10	62 M	Indian	Renal	-ve	239	25	2.98	S/K
11	68 F	Indian - Punjabi	Renal	-ve	186	24	2.97	S/K/A
12	75 F	Indian	Renal	-ve	183	25	0.10	L/S/K/A
13	69 M	Pakistani	Renal	-ve	178	34	0.10	L/S/K
14	66 M	Pakistani	Renal	-ve	424	HD	0.12	A
15	67 F	Pakistani	Renal	-ve	284	15	1.06	S
16	59 F	Pakistani	Renal	-ve	131	38	0.20	S/K/A
17	55 M	Pakistani	Renal	-ve	127	53	0.51	S/K
18	75 M	Pakistani - Kashmiri	Hepatic	B & C	114	56	0.10	L/S/K/A
19	70 M	Pakistani - Punjabi	Renal	-ve	213	20	0.65	S/K
20	71 F	Punjabi	Renal	B	143	32	1.74	S/K
21	53 M	Sudanese	Hepatic	C	80	93	0.10	S
22	54 F	Sudanese	Renal	-ve	562	HD	Anuric	L/S/K
23	56 F	Mexican	Rectal	C	57	100	0.10	L/S
24	73 F	Indian	Renal	-ve	206	21	0.18	L/S/K

Median age at diagnosis was 62 years (range 37–75), and male to female ratio was roughly equal (14/10). Notably, 16 (67%) patients were from the Indian subcontinent (India and Pakistan), 7 (30%) patients were of Middle Eastern ancestry (Egyptian and Sudanese), and one was of Mexican origin. ALECT2 amyloidosis was not diagnosed in any British Caucasians. Patient demographics at diagnosis are shown in Table 4.1.

Two patients had established ESRD and were receiving renal replacement therapy in the form of hemodialysis at the time of diagnosis of ALECT2 amyloidosis. Neither had presented with nephrotic syndrome. Among the remaining 22 patients who remained dialysis independent at the time of diagnosis, median eGFR was 33 ml/min (range 15-110) and median proteinuria was 0.5 g/24 hr (range 0.1-2.9).

Liver function tests were entirely normal among 21 patients. There was mild elevation of transaminases (ALT 54 and 73 U/L) in 2 patients and mildly obstructive liver function in a single patient as evidenced by an alkaline phosphatase of 150 U/L. No patient had hyperbilirubinaemia or clinical evidence of jaundice. No patient had clinical, echocardiographic or cardiac biomarker evidence of cardiac amyloidosis or clinical evidence of amyloid peripheral or autonomic neuropathy.

None of the 24 patients had evidence of a monoclonal gammopathy. Four patients had a concurrent diagnosis of type 2 diabetes mellitus.

Histology

Renal histology revealed amyloid throughout the kidneys, often with bright congophilia and ‘sparkly glistening’ apple green birefringence when viewed under crossed polarized light. Liver histology revealed amyloid deposits with a similar ‘sparkly glistening’ appearance under crossed polarized light.

Among 20 patients who underwent renal biopsies, the vast majority (85%) had amyloid in the renal cortical interstitium which was sometimes extensive and often associated with tubular atrophy. Amyloid deposits were identified in the tubular basement membranes in approximately one third of renal biopsies and in the vessel walls in two thirds (Table 4.2). Glomerular amyloid was

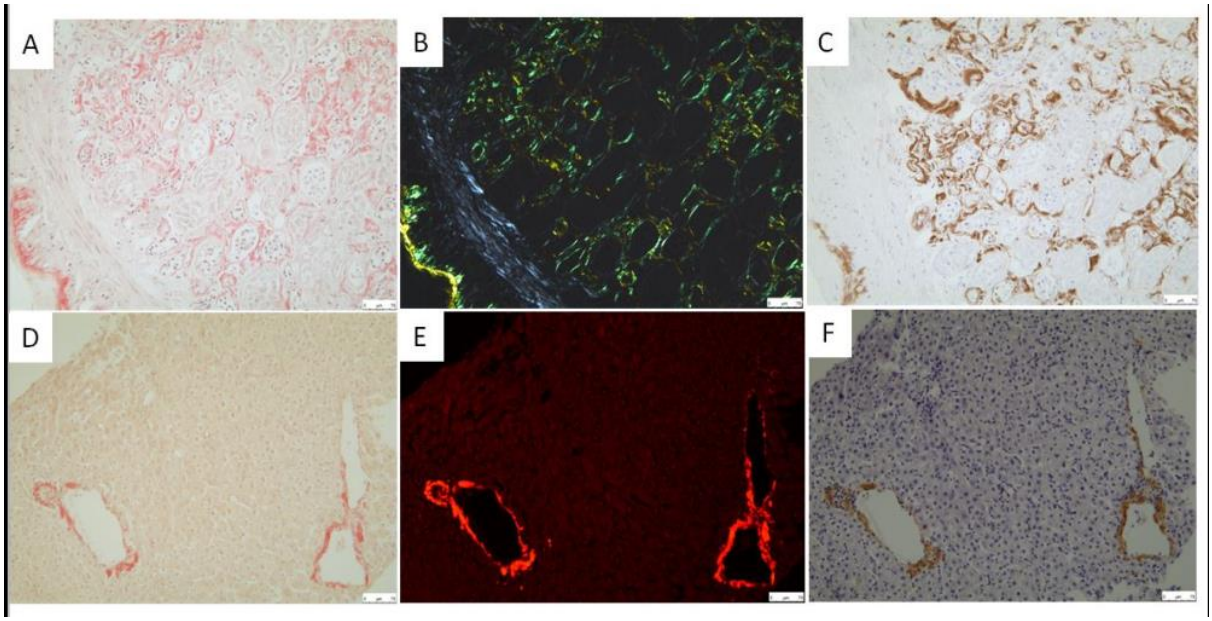
present in more than 70% of renal biopsies, and was associated with mesangial matrix expansion which was usually diffuse and global (Figure 4.1A and 4.1B). Typically, a proportion of biopsied glomeruli were globally sclerosed. The burden of amyloid within each compartment of the kidney varied substantially between patients, and apart from the extensive interstitial amyloid observed in some patients, there was no 'characteristic' pattern of deposition or apparent relationship between ethnicity and localization of renal amyloid deposits.

Table 4.2 Renal histology in relation to clinical presentation in ALECT2 amyloidosis

Patient Number	**Interstitial Amyloid (+/-)**	**Glomerular Amyloid (+/-)**	**Tubular Amyloid (+/-)**	**Vascular Amyloid (+/-)**	**Diabetes (Y/N)**	**eGFR (ml/min)**	**24 urinary protein loss (g/24hr)**	**Hypertension (Y/N)**
1	+	-	-	-	0	30	0.17	N
2	+	+	-	-	0	24	0.10	N
3	+	+	-	+	0	44	0.47	N
4	+	-	+	-	0	27	0.48	N
6	+	+	+	+	0	15	0.90	Y
7	+	-	-	+	0	110	0.10	N
8	+	+	-	+	0	33	0.10	N
9	-	+	+	+	0	40	2.52	Y
10	+	-	-	+	1	25	2.98	N
11	-	+	+	+	1	24	2.97	Y
12	-	-	-	+	0	25	0.10	Y
13	+	+	-	+	0	34	0.10	N
14	+	+	-	+	1	HD	0.12	N
15	+	+	-	+	0	15	1.06	Y
16	+	+	-	+	0	38	0.20	Y
17	+	+	-	-	0	53	0.51	N
19	+	+	-	-	0	20	0.65	Y
20	+	+	-	-	0	32	1.74	Y
22	+	+	-	+	0	HD	Anuric	Y
24	+	+	+	+	1	21	0.18	N

Liver histology revealed amyloid deposits in vessel walls and globular deposits in the portal tracts (Figure 4.1D and 4.1E). Gastrointestinal histology showed amyloid in the submucosa. The amyloid stained specifically with anti-LECT2 antibody in every case (Figure 4.1C and 4.1F).

Figure 4.1 Histology in ALECT2 amyloidosis:



A) Congo red staining of a renal biopsy specimen showing amyloid in all renal compartments including the glomeruli, renal tubules, vessels and interstitium. B) Apple green birefringence when viewed under cross polarized light. C) Immunohistochemical staining with an antibody against LECT2 showing specific staining of the amyloid which is completely abrogated by prior absorption of the antibody with purified LECT2. D) Congo red staining of a liver biopsy specimen viewed under Brightfield light showing amyloid in vessels and portal tract. E) Characteristic immunofluorescence of amyloid after Congo red staining and F) Immunohistochemical staining with an antibody against LECT2 showing specific staining of the amyloid.

Proteomic Analyses

Proteomic analysis of the excised amyloid was performed in 14/24 samples. LECT2 was identified in 12/14 cases; there was insufficient material for valid interpretation of proteomic results in the two negative cases, each of which had <30 proteins identified in the sample (both of whom were

diagnosed on the basis of immunohistochemistry). A positive control sample of authentic LECT2 treated in the same way as the patient samples identified LECT2 with 95% protein coverage.

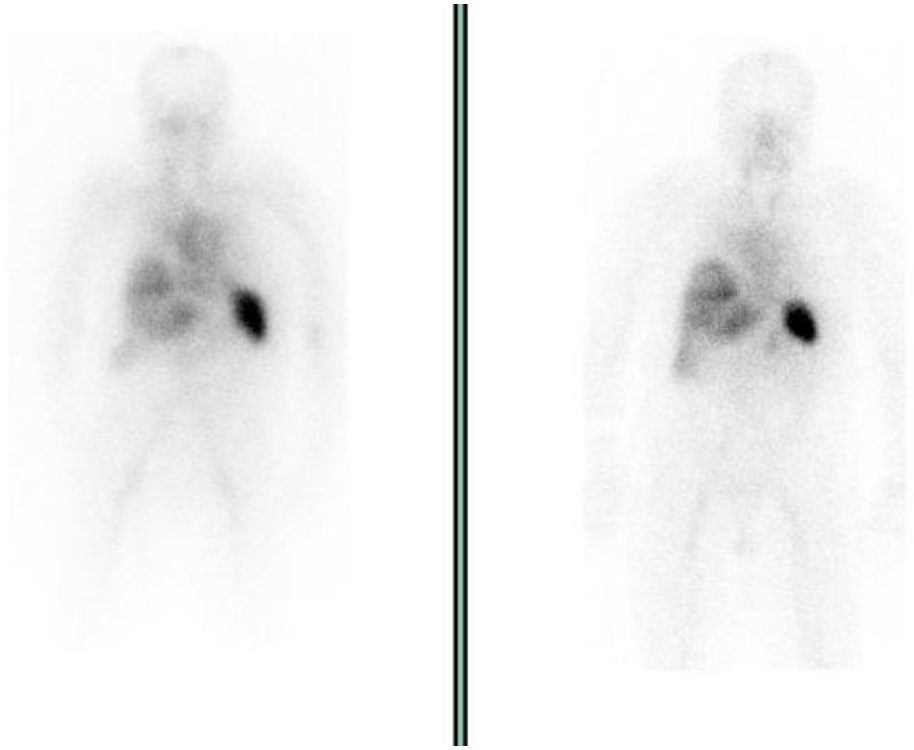
Genetic Analyses

All patients underwent genetic analysis and all except one affected individual were found to be homozygous for the G allele, a polymorphism which results in a substitution of isoleucine with valine at position 40 in the mature protein. The remaining affected individual was heterozygous for the G allele, which as far as the authors are aware, is the first report of ALECT2 amyloidosis in a patient who is heterozygous for this allele.

^{123}I-Labelled SAP Scintigraphy

SAP scintigraphy at diagnosis showed extra-renal amyloid deposits in most patients; 21/24 (88%) had splenic amyloid, 11/24 (46%) had hepatic amyloid and 9/24 (38%) had adrenal gland involvement by amyloid despite preserved adrenocortical function in every case. The adrenal glands were masked by heavy overlying liver and spleen amyloid deposits in 8 of the 15 remaining cases. Serial SAP scintigraphy, with an interval between the diagnostic and latest scan of up to 10 years, revealed little change in total body amyloid burden. There was no evidence of amyloid regression from any organ in any patient but, interestingly and uncharacteristically for untreated systemic amyloidosis, nor was there evidence of accumulation of amyloid within the liver, kidneys or spleen over prolonged periods (Figure 4.2)

Figure 4.2: Serial SAP scintigraphy in ALECT2 amyloidosis. SAP anterior whole body SAP scans, taken 10 years apart, in a patient with ALECT2 amyloidosis showing absence of amyloid accumulation in the liver or spleen over this time period.



Renal and Patient Outcomes

Median follow up in the whole cohort was 4.8 years (range 0.5–15.2). During this time, 4 patients died. The cause of death was known in 3 cases who died from bronchopneumonia, cardiac arrest secondary to underlying ischaemic heart disease (IHD), and 'pump failure' secondary to aortic stenosis respectively. Median age at death was 74 years (range 63–77). Median estimated patient survival from diagnosis of ALECT2 amyloidosis was 15.1 years (Figure 4.3a).

Among 22 patients who were dialysis independent at diagnosis, mean rate of GFR loss was 4.2 ml/min/yr (range 0.5-9.6). Four patients progressed to ESRD during follow up. Median estimated time from diagnosis to ESRD by Kaplan Meier analysis among those 22 patients was 8.2 years

(Figure 4.3b). Proteinuria remained sub-nephrotic and serum albumin remained within the normal range in all patients throughout follow up. Indeed there was no significant change in proteinuria over time among most patients (mean change <0.1 g/24hr/yr).

Figure 4.3a: Outcome in ALECT2 amyloidosis. a) Patient survival in ALECT2 amyloidosis by Kaplan Meier analysis. Median estimated survival was 15.2 years.

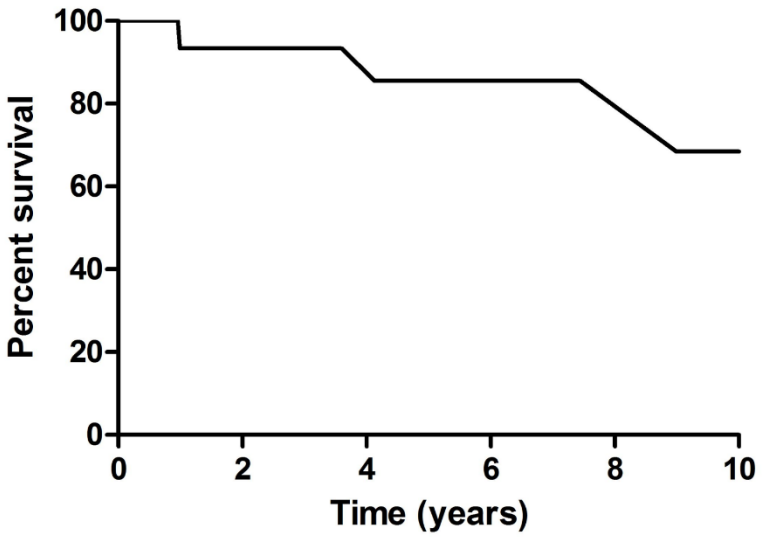
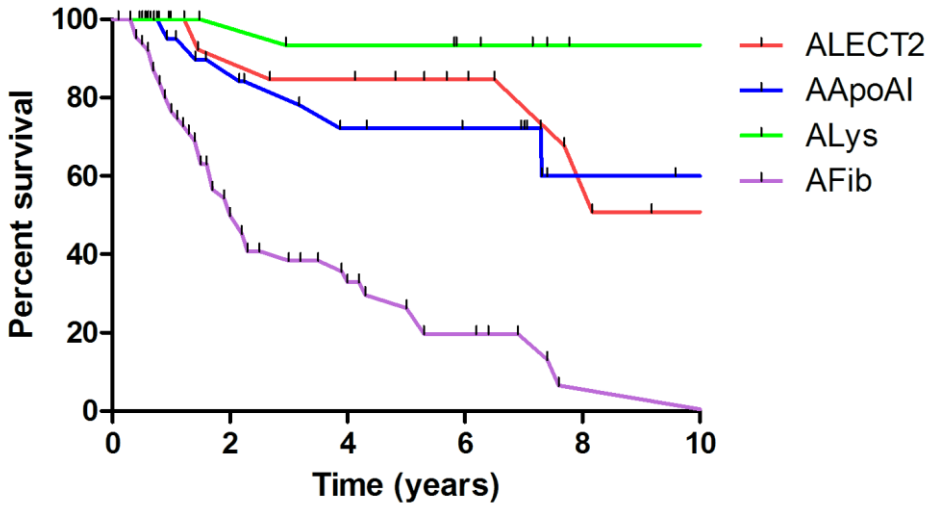


Figure 4.3b: Outcome in ALECT2 amyloidosis. b) Renal survival, defined as time from diagnosis to renal replacement therapy, in ALECT2 amyloidosis in comparison to other untreated renal amyloidoses including AApoAI, ALys, and AFib amyloidosis.



Discussion

LECT2 was reported to be a new amyloid fibril protein by Benson and colleagues in 2008 following discovery of unanticipated amyloid within a nephrectomy specimen from an individual with co-existing clear cell renal carcinoma.(137) LECT2 is a 16 kDa protein consisting of 151 amino acids, including an 18 amino acid signal peptide. The amyloidogenic fibril protein in ALECT2 amyloidosis is composed of the entire 133-residue peptide. Although there is no mutation in the *LECT2* gene associated with ALECT2 amyloidosis, nearly all affected individuals in our series, including two affected first degree relatives, were found to be homozygous for the G allele, a polymorphism which results in a substitution of isoleucine with valine at position 40 in the mature protein, consistent with previous reports.(137) The G/G genotype has an overall frequency of 0.477 and in subjects of European descent it is found at a frequency range of 0.6-0.7,(137) although the frequency of this polymorphism in the studied population is unknown. Murphy *et al* described a clear ethnic

distribution in their case series of ten patients with ALECT2 amyloidosis; 7 of the 10 patients were of Mexican American origin,(138) a finding corroborated by the Nephropath investigators who found that the majority of patients with ALECT2 amyloidosis were of Hispanic origin.(146) A recent biopsy series revealed ALECT2 amyloid to be the second commonest type of renal amyloid in Egypt, accounting for almost one third of cases.(147) Our cohort of 24 patients further supports an ethnic bias but includes individuals from the Indian Subcontinent and Egypt/Sudan as well as Hispanics, corroborating the recent findings of Larson et al.(146)

The aetio-pathogenetic mechanisms underlying ALECT2 amyloid formation require further evaluation. LECT2 is mostly produced by the liver and is known to be a chemotactic factor to neutrophils while it also stimulates the growth of chondrocytes and osteoblasts.(148) Lin Bin *et al* identified LECT2 as an acute phase reactant with a high level of induction upon infection,(149) and it appears to play a role in liver regeneration.(150) One might speculate therefore, that LECT2 is an acute phase reactant that is specifically triggered by a chronic hepatic infection or chronic hepatic inflammation and that the combination of the G/G genotype and excessive production of LECT2 by the liver predispose to development of amyloid. Other proposed mechanisms for ALECT2 amyloidosis included interference in the catabolic or transport pathways of LECT2 resulting in an increased local tissue concentration or alterations in a binding partner of the LECT2 protein in the serum resulting in increased free circulating LECT2 levels.(141) Due to the absence of a commercially available LECT2 assay, we were unable to measure plasma LECT2 concentration among our patient cohort in order to further explore these possibilities.

The vast majority of patients diagnosed with ALECT2 amyloidosis had a renal presentation characterized by low level proteinuria and a reduced GFR. Given the frequent (>70%) glomerular involvement by amyloid in this cohort, the absence of heavy proteinuria is surprising, and contrasts other types of renal amyloid in which the glomeruli are involved which are typically characterized by the nephrotic syndrome. Around half of patients with ALECT2 amyloidosis had evidence of liver involvement by SAP scintigraphy but liver synthetic function was preserved in every case and liver function abnormalities were absent or mild, consistent with previous reports.(139) Three patients were diagnosed on liver histology, prompted in each case by another potential cause of liver

dysfunction; presence of amyloid may have been incidental to the dysfunction since amyloid deposits were frequently present among those without derangement of liver function. No patient had cardiac amyloidosis at diagnosis and clinically significant pulmonary or neural amyloid was not observed.

Staining of formalin fixed paraffin embedded renal and liver sections with an anti-LECT2 antibody was unequivocally diagnostic in every case. There was no background or non-specific staining of the sections, and the amyloid deposits were avidly and specifically stained by the antibody in both tissues. The immunohistochemistry was corroborated by proteomic analysis of amyloid whenever this was undertaken on adequate samples. The authors would therefore recommend that tissue from all cases of suspected ALECT2 amyloidosis is stained immunohistochemically and/or analyzed by tandem mass spectrometry.

Despite absence of amyloid-specific therapy for ALECT2 amyloidosis, its natural history, described here in detail for the first time, is slow. Mean rate of GFR loss was 4.2 mls/min/year contrasting the typical decline in other types of renal amyloidosis (Table 4.3), such as untreated renal AL amyloidosis in which median time from diagnosis to ESRD is only about 12 months;(151) only 6 patients in the whole cohort reached ESRD despite prolonged follow up. Serial SAP scans, performed up to a decade apart, revealed absence of amyloid accumulation and similarly, no patient developed cardiac or neuropathic amyloid during follow up. Consequently, median estimated patient survival from diagnosis was more than 15 years.

Given the discrepancy between the clinical and histological prevalence of ALECT2 amyloid and the often insidious nature of its clinical manifestations, (142) the true prevalence of this type of amyloid may be greater than is currently recognized. Since promising novel therapies to remove existing amyloid deposits are now under development,(25) the diagnosis of ALECT2 amyloidosis ought to be considered in any non-Caucasian patient with renal amyloid deposits who has low level proteinuria, even when a monoclonal protein is present.

Table 4.3. Comparison of rate of GFR loss in ALECT2 amyloidosis and hereditary renal amyloidosis

Type of Amyloid	**Number**	**Mean eGFR loss per year ml/min/year**
Leucocyte chemotactic factor 2 amyloidosis (ALECT2)	22	4.2
Lysozyme (ALys)	7	4.7
Apolipoprotein A-1 (AApoAI)	24	6.2
Fibrinogen A α-chain (AFib)	23	11.5(46)

Chapter Five: Prolonged renal survival in light chain amyloidosis: speed and magnitude of light chain reduction is the crucial factor.

Introduction

The amyloidoses are disorders of protein folding, in which a variety of proteins misfold and aggregate into amyloid fibrils that accumulate in tissues and disrupt organ function.(152) Immunoglobulin light chain (AL) amyloidosis is caused by deposition of fibrils derived from monoclonal immunoglobulin light chains and is the most common and serious type of systemic amyloidosis.(5) Renal involvement is present in approximately 70% of patients with systemic AL amyloidosis at diagnosis, manifesting with nephrotic syndrome and progressive renal impairment.(101) Progression to end-stage renal disease (ESRD) is one of the main determinants of morbidity in AL amyloidosis,(153) whilst presence and severity of cardiac amyloidosis is the main determinant of mortality.(67, 68)

Response to chemotherapy has been shown to be strongly and independently associated with both patient survival(101) and renal outcomes in patients with AL amyloidosis.(130, 154) Other factors associated with poor renal outcomes in those with renal AL amyloidosis include a low GFR and heavy proteinuria at diagnosis.(130, 155) However, there are no data on whether chemotherapy can delay onset of renal replacement therapy in patients with AL amyloidosis who present with established advanced CKD and whether speed of clonal response influences renal outcome.

The AL Amyloidosis Chemotherapy study (ALchemy) is a comprehensive prospective observational study opened in 2009 into which all patients newly diagnosed with AL amyloidosis at the UK National Amyloidosis Centre (NAC) are invited to participate. We report here the renal and patient outcomes among all participants in ALchemy who had advanced CKD at the time of

diagnosis (and entry to study) between 2009 and 2015 in relation to the speed and depth of the hematologic response to chemotherapy.

Patients and Methods

Patients

At the time of censor, 1000 patients with newly diagnosed AL amyloidosis had been enrolled into the ALchemy prospective observational study at the National Amyloidosis Centre (NAC). Renal involvement, defined as non-Bence Jones proteinuria of more than 0.5g/24 hr according to the amyloidosis international consensus criteria,(69) was present in 672 patients, of whom 84 had presented with advanced renal impairment defined by eGFR <20 ml/min/1.73 m^2. The analyses presented in this manuscript concern this cohort of 84 patients with eGFR <20 ml/min/1.73 m^2 at baseline (Figure 5.1; Consort Diagram).

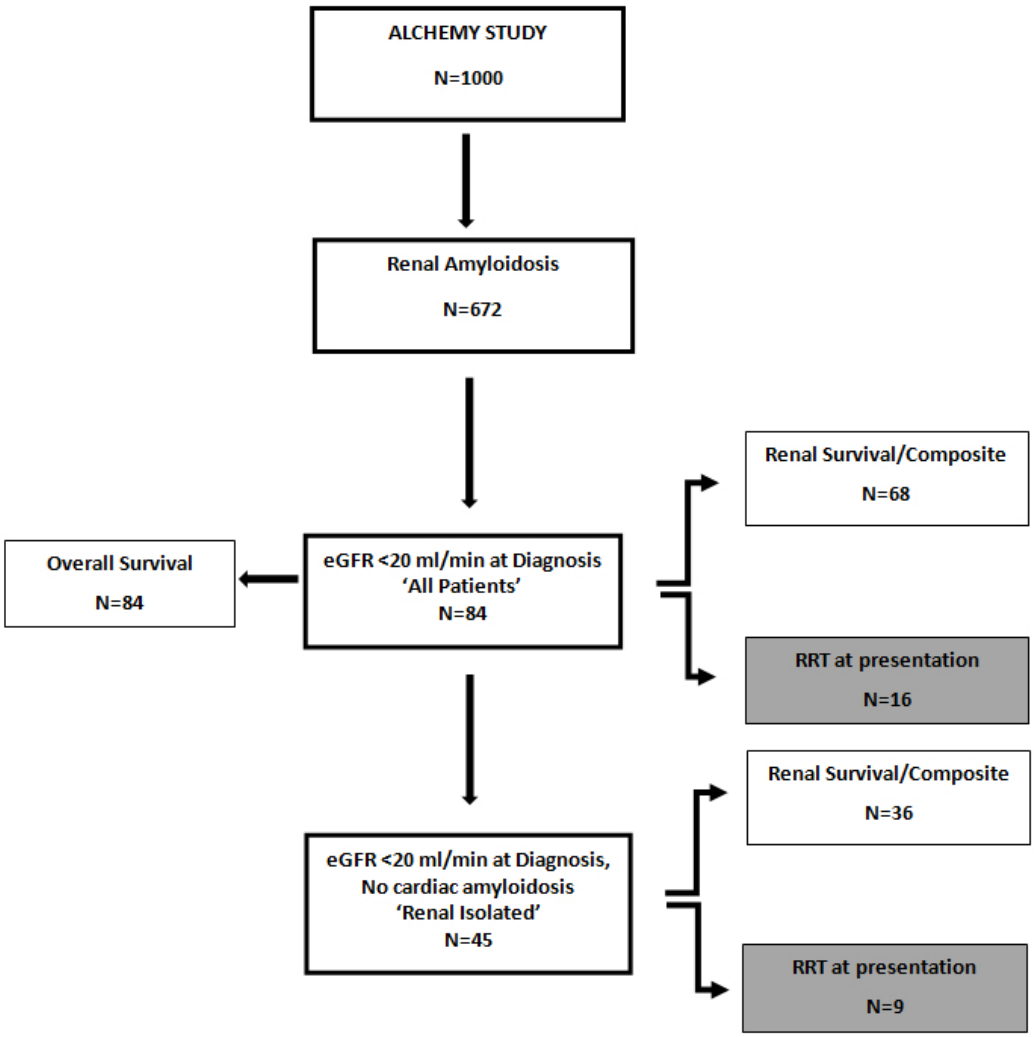
All patients underwent protocolized assessments every 3-6 months at the NAC, each assessment comprising clinical evaluation, serum and urine biochemistry including assessment of renal and liver function, N-terminal pro-b-type natriuretic peptide (NT-proBNP), echocardiography, SAP scintigraphy,(65) and assessment of hematological disease by serum free light chain (FLC) assay, serum and urine immunofixation electrophoresis. The presence of cardiac amyloidosis was defined by echocardiography according to international consensus criteria,(69) or in cases in which there was doubt, by additional cardiac magnetic resonance imaging on the basis of native T1 and/or extracellular volume measurement, as previously reported.(156, 157)

All patients were managed in accordance with the Declaration of Helsinki and provided written informed consent for study entry (REC reference 09/H0715/58) and publication of their data.

Renal Histology

Renal biopsies were performed in 57 of 84 patients. All biopsies were routinely stained with Congo red and a panel of amyloid-fibril antibodies, as previously described.(114) Additionally, all biopsies containing sufficient cortical tissue for evaluation (n=49/57) were analysed by a renal histopathologist (PB) and assigned an 'Index of Chronic damage' category of mild, moderate or severe according to the previously described Modified Oxford Score.(158)

Figure 5.1 Consort Diagram showing selection of patients from the prospective UK AL chemotherapy study (ALchemy) for analyses. Patients in shaded boxes were excluded from analyses of renal survival.



Assessment of Hematologic Response

Details and doses of chemotherapy regimens were collected. All patients had serial FLC concentration prospectively monitored on blood samples scheduled monthly during periods of chemotherapy treatment, and every 1-3 months during subsequent follow up. Healthy polyclonal serum FLC concentrations increase progressively through advancing stages of chronic kidney disease (CKD)(122) which hinders the monitoring of monoclonal light chain disorders. In this study, the value of the FLC monoclonal component was estimated by subtracting the concentration of the uninvolved light chain from that of the amyloidogenic light chain to obtain the FLC difference (dFLC), a strategy previously validated in multiple myeloma and AL amyloidosis.(123, 130)

The FLC response to chemotherapy was determined according to previously validated 'consensus criteria'(159) and additionally, by the percentage of the baseline dFLC that remained at the time of analysis (percentage method), also validated in AL amyloidosis.(130) The consensus criteria define 'evaluable' patients as those with a pre-treatment (baseline) dFLC of >50 mg/L, and thus excluded 10/84 (12%) patients in the cohort, whereas the calculation of the percentage baseline dFLC remaining after chemotherapy can be applied to patients with low level pre-treatment amyloidogenic light chain concentration. A very good partial response (VGPR) was defined according to the consensus criteria as an absolute dFLC of <40mg/L, and by the percentage method as a ≥90% reduction of pre-treatment dFLC remaining after chemotherapy, as previously described.(130) When assessing dFLC response, all patients without an FLC assay at the relevant timepoint were excluded from analysis.

Patient Outcomes

Overall survival was defined as the time from baseline evaluation at the NAC to patient death and was evaluated in all 84 patients. Renal survival was defined as the time from baseline evaluation at the NAC to requirement for renal replacement therapy (RRT). For the analyses of renal survival, patients who were already established on RRT (n=16) at the time of their baseline

evaluation were excluded, and those who died without requiring RRT were censored at the time of death. For analyses of time to the composite endpoint of death or dialysis, patients who were on RRT at baseline were excluded and an event was recorded as the first of either death or dialysis. Patient follow up was censored on 1st October 2015.

Statistical Analysis

Survival analysis was performed separately for each of three possible endpoints: patient survival, renal survival, and survival to composite endpoint of dialysis or death. We determined Kaplan-Meier curves, and performed the log rank test to compare the overall survival curves for different subgroups. Cox proportional hazards regression analysis was used to investigate the factors independently associated with a particular endpoint. A test based on Schoenfeld residuals was used to test the proportional hazards assumption underlying the log rank and the Cox regression analyses. Analyses were performed using GraphPad Prism v5.03, IBM SPSS Statistics 23 and Stata 14 software. A significance level of 0.05 was used for all hypothesis tests.

Results

Baseline Characteristics and Patient Survival

Baseline demographics and clinical characteristics of all 84 patients are listed in Table 5.1. The cohort was followed for a median of 16.3 months (range 0.4-68.0) from baseline. Forty five of 84 patients had 'renal isolated' involvement and 39 had evidence of both cardiac and renal involvement (Figure 5.1). Fifty-seven of 84 patients had renal histology performed and all biopsies showed extensive renal infiltration by amyloid; the only other notable pathology being hypertensive arteriosclerosis in 2 cases. Median age at diagnosis in the whole cohort was 68 years with an almost equal male to female ratio. Median eGFR was 10 ml/min/1.73 m^2 with a median 24 hour urinary protein leak of 6.2 grams. Serum albumin was modestly reduced with a median of 31 g/L despite substantial proteinuria in the majority of cases. Median NT-proBNP was

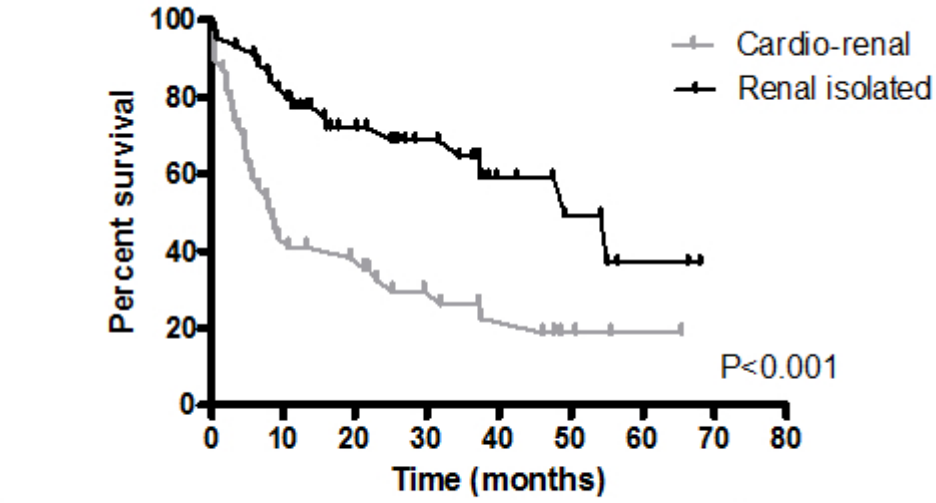
550 pMol/L with 32 patients having a concentration >1000 pMol/L (i.e., Mayo stage 3b disease).(68)

A total of 47/84 (56%) patients from the whole cohort died with median time from baseline to death by Kaplan Meier analysis of 25.2 months (CI 9.4-41.1). Thirty of 39 patients with cardio-renal syndrome (due to cardiac and renal involvement by amyloid) died and 17 of 45 patients with 'renal isolated' amyloidosis died. Median overall survival was significantly longer (49.2 months [CI 34.5-undefined]) among those with 'renal isolated' amyloidosis compared to those with cardio-renal syndrome (8.4 months [CI 4.7-22.9], $p<0.001$) (Figure 5.2). Cause of death among those with cardio-renal syndrome was invariably from progressive cardiac amyloidosis, and in those with renal isolated amyloidosis, was from 'progressive amyloidosis' in 9 cases, and from sepsis, cerebrovascular accident, and incarcerated femoral hernia in one case each. In 5 cases the cause of death was unknown.

Table 5.1. Baseline demographics of all patients

Demographic or clinical characteristic		**No. of Patients (N =84)**	**%**
Male sex Female sex		49 35	58 42
Age years	Median Range	68 40-86	
Patients with isolated renal involvement		45	54
Patients presenting on RRT		16	19
Index of Chronic Damage on renal histology	Mild Moderate Severe	4/49 11/49 34/49	8 22 69
eGFR (ml/min/1.73 m^2)	Median Range	10 10-19	
Amyloid load by SAP scintigraphy	Small Moderate Large	27 19 38	32 23 45
Serum albumin (g/L)	Median Range	31 14-48	
24hr urinary protein loss (g)	Median Range	6.2 0.1-29.7	
NT-proBNP (pMol/L)	Median Range	550 15-8270	
Troponin T (ng/L)	Median Range	120 10-1870	
Amyloidogenic light chain (n)	Lambda Kappa	55 29	65 35
Haemoglobin (g/dL)	Median Range	11.3 7.8-17	
Serum creatinine (µmol/L)	Median Range	375 228-979	
Bilirubin (µmol/L)	Median Range	5 1-59	
Alkaline Phosphatase (u/L)	Median Range	108 43-1703	
Supine systolic blood pressure	Median Range	137 79-184	
Standing systolic blood pressure	Median Range	129 63-180	
Bone Marrow Plasmacytosis (%)	Median Range	7 0-30	
Bence Jones Protein (n)	Present Absent	44 40	
λ sFLC in AL (lambda) patients (mg/L)	Median Range	241 14-5820	
K sFLC in AL (kappa) patients (mg/L)	Median Range	431 56-10300	

Figure 5.2. Patient survival calculated by Kaplan Meier analysis in all evaluable patients with renal AL amyloidosis and eGFR <20 ml/min/1.73 m^2 at presentation. Survival among those with 'renal isolated' amyloidosis was significantly longer (median 49.2 months) than in those with both cardiac and renal (cardio-renal) involvement (median 8.4 months) (p<0.001). Number at risk at certain timepoints is shown in panel below graph.



Renal	39	36	24	17	8	5	2	0
Cardio-renal	45	17	14	9	6	3	1	0

Chemotherapy

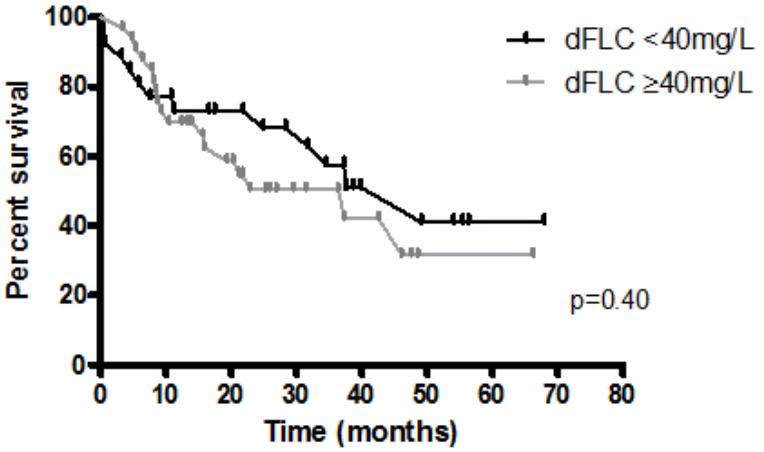
Chemotherapy was planned in all 84 cases, but was actually administered to 78 patients. Reasons for non-administration of chemotherapy were patient death from progressive amyloidosis in 4/6 patients, and dialysis-dependence in 2 patients who did not have significant extra-renal amyloid. Among those who did receive chemotherapy, 43 (55%) received bortezomib-based regimens first line, 22 (28%) received thalidomide-based regimens first line, and 13 (17%) received a first line regimen containing neither bortezomib nor thalidomide. Median (range) number of chemotherapy cycles administered first line was 4 (1-8) for each of bortezomib-based, thalidomide-based and non-bortezomib, non-thalidomide containing regimens. No patient discontinued bortezomib therapy, but 1 patient discontinued thalidomide, and 1 patient discontinued non-bortezomib, non-thalidomide chemotherapy due to toxicity. As-treated analysis of the 78 patients who received chemotherapy showed a dFLC response at 3 months of ≥90% in 15/43 (34%) who received bortezomib compared to 4/22 (18%) who received thalidomide (bortezomib vs thalidomide, p=0.09, Fisher's exact test) and 2/13 (15%) who received neither drug first line (bortezomib vs neither bortezomib nor thalidomide, p=0.12, Fisher's exact test).

Overall survival (OS) in relation to response to chemotherapy

Seventy-four patients were evaluable for dFLC response by consensus criteria (i.e., had a dFLC at baseline of >50mg/L). Of those 74 patients, 15 did not have a dFLC measurement at 3 months, in 11 cases due to prior death, and were therefore excluded from the analysis of survival in relation to hematologic response at this timepoint. Of the 11 patients who died, 6 did so before receiving chemotherapy, 4 died from progressive amyloidosis and one died from chemotherapy-related complications (sepsis). There was no significant difference in overall survival between 26 evaluable patients who achieved a dFLC of <40mg/L within 3 months of baseline (median 49.2 months [CI 25.0–undefined]) and 33 evaluable patients who achieved lesser degrees of clonal response at the same timepoint (median 37.4 months [CI 11.3–undefined]) (log rank test, p=0.40) (Figure 5.3a). Using the 'percentage of baseline dFLC' method to calculate hematologic response enabled all patients to be considered 'evaluable' at baseline although, as described

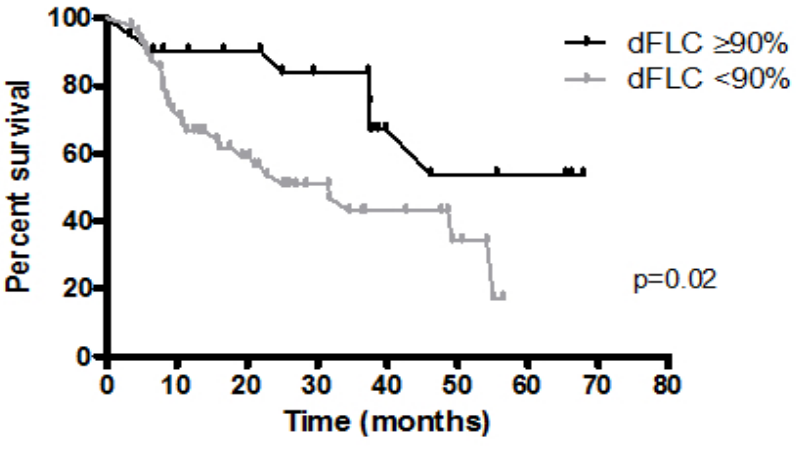
above, the 15 patients who did not have a dFLC measurement at 3 months were again excluded from this analysis. Median overall survival among 21 evaluable patients who achieved a dFLC response of ≥90% within 3 months of baseline was undefined compared to 31.8 months (CI 15.7–55.1) among 48 patients who achieved lesser degrees of clonal response at the same timepoint (log rank test, p=0.02) (Figure 5.3b). There was no significant difference in overall survival between those patients who achieved a <50% dFLC response at 3 months and those who achieved a dFLC response of 50-89% (log rank test, p=0.09) (Figure 5.3c).

Figure 5.3. Patient survival calculated by Kaplan Meier analysis in all evaluable patients with renal AL amyloidosis and eGFR <20 ml/min/1.73 m^2 at presentation. A) Patients were stratified according to degree of clonal response at 3 months into absolute dFLC <40 mg/L and ≥40 mg/L (p=0.40). B) Patients were stratified according to degree of clonal response at 3 months into dFLC response ≥90% and dFLC response <90% (p=0.02). C) Patients were stratified according to degree of clonal response at 3 months into dFLC response of <50% and 50-89% (p=0.09). Number at risk at certain timepoints is shown in panel below graph.



<40	26	23	21	17	14	6	2	0
≥40	33	32	25	17	9	6	2	0

Figure 5.3a



≥90%	21	19	16	13	6	5	4	0
<90%	48	36	24	15	10	5	0	0

Figure 5.3b

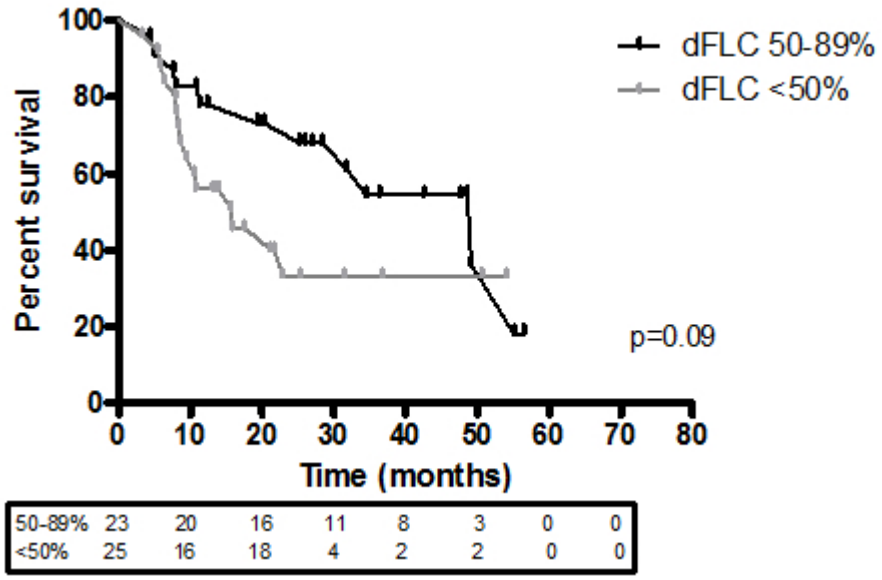


Figure 5.3c

By Cox regression analysis, independent factors associated with death in the whole cohort of 84 patients were elevated NT-proBNP at presentation (HR 2.72 [CI 1.451-5.088], p=0.002) and achieving a dLFC response ≥90% at 3 months (HR 0.36 [CI 0.138-0.935], p=0.036). Percentage dFLC response was also highly significant when incorporated as a continuous variable (HR 0.980 [CI 0.968-0.992], p=0.001)

Renal survival in relation to response to chemotherapy

Among 68 patients who were dialysis-independent at baseline and therefore evaluable for analyses of renal survival, there were 46 patients who were evaluable for hematologic response at 3 months according to consensus criteria. Among 22/46 who achieved an absolute dFLC of <40mg/L within 3 months of baseline, median time to dialysis dependence was 9.7 months (CI 3.4–undefined) compared to 5.2 months (CI 1.9-17.1) among 24/46 patients who achieved lesser degrees of clonal response at the same timepoint (log rank test p=0.18) (Figure 5.4a). Using the 'percentage of baseline dFLC' method to calculate hematologic response, there were 56 patients who were evaluable for hematologic response at 3 months. Median renal survival among 18/56 patients who achieved a dFLC response of ≥90% within 3 months of baseline was 23.0 months (CI 9.7–undefined) compared to 6.1 months (CI 3.4-12.5) among 38/56 patients who achieved lesser degrees of clonal response at the same timepoint (log rank test, p=0.003) (Figure 5.4b).

Renal outcomes were equally poor among those who achieved a delayed ≥90% dFLC response, classified as only after 6 months from baseline (n=5) or only after 12 months from baseline (n=6) (dFLC response ≥90% within 3 months vs 6 months (log rank test, p=0.001) or vs 12 months (log rank test, p<0.003) (Figure 5.4c).

Figure 5.4. Renal survival calculated by Kaplan Meier analysis in all evaluable patients with renal AL amyloidosis and eGFR <20 ml/min/1.73 m^2 at presentation. A) Patients were stratified according to degree of clonal response at 3 months into absolute dFLC <40 mg/L and ≥40 mg/L (p=0.18). B) Patients were stratified according to degree of clonal response at 3 months into dFLC response ≥90% and dFLC response <90% (p=0.003). C) Patients were stratified according to speed of clonal response comparing those who achieved a dFLC ≥90% within 3 months of baseline with those who achieved an equally good dFLC response, but only after 12 months (p<0.003). Number at risk at certain timepoints is shown in panel below graph.

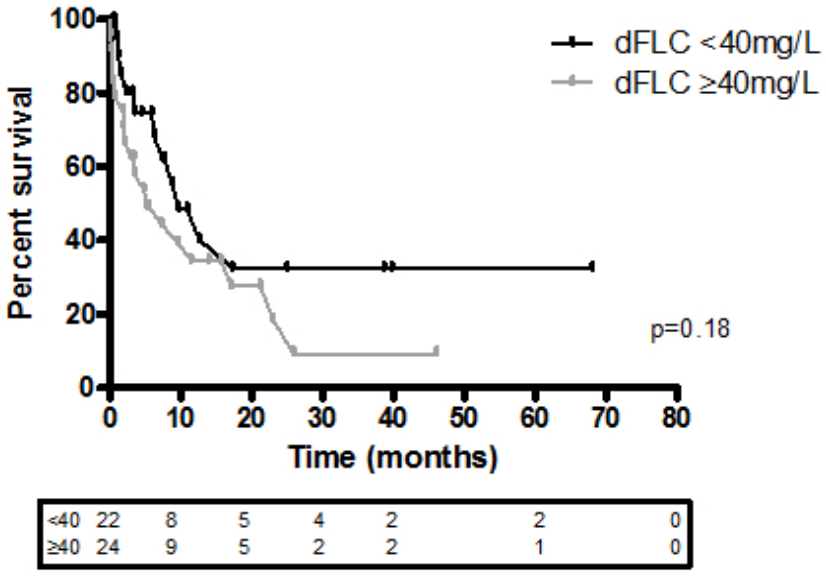


Figure 5.4a

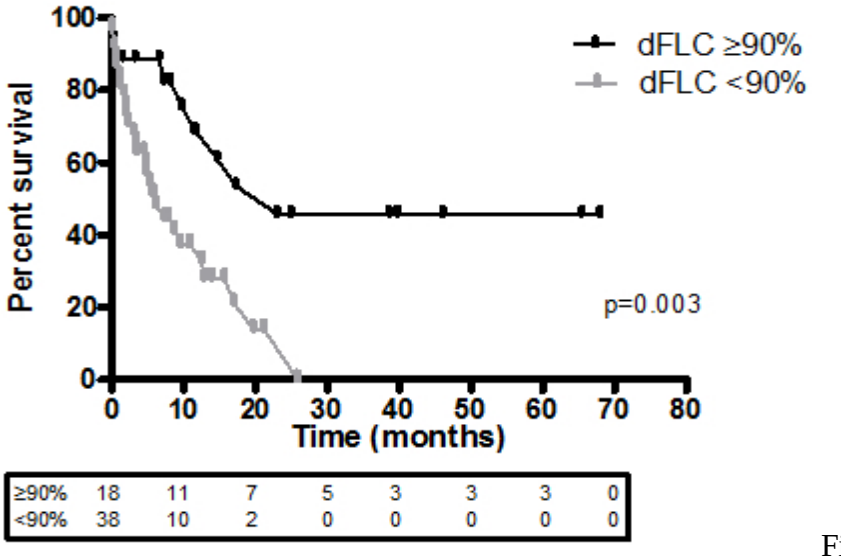


Figure 5.4b

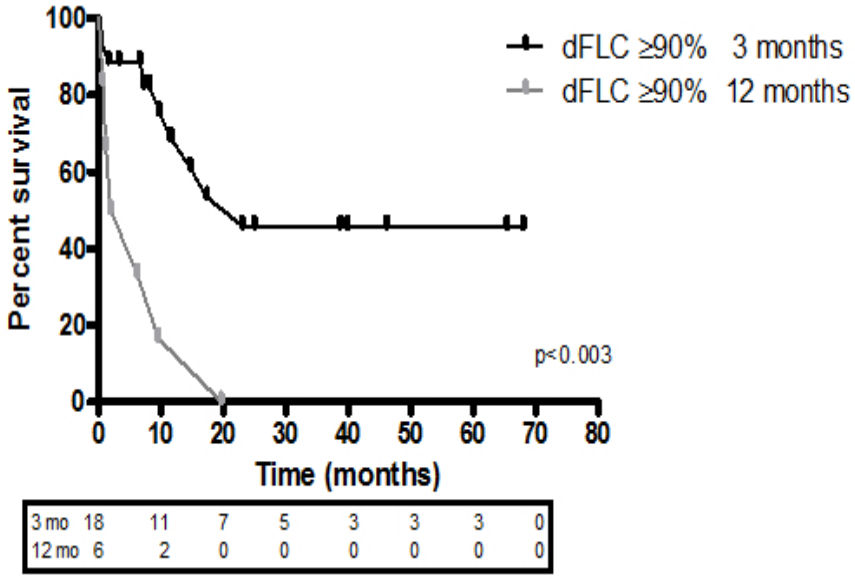


Figure 5.4c

By Cox regression analysis, the only independent factor associated with a requirement for RRT among the 68 patients who were dialysis independent at baseline was achieving a dFLC response of ≥90% within 3 months (HR 0.24 [CI 0.106-0.547], p=0.001) (Table 5.2). Percentage dFLC response at 3 months was also significant when incorporated as a continuous variable (HR 0.978 [CI 0.958-0.998], p=0.031). Interestingly, presenting eGFR, presenting NT-proBNP, and proteinuria at presentation did not predict progression to dialysis. Furthermore, stratification of patients by index of chronic damage on renal histology was not predictive of progression to dialysis, although it should be noted that the vast majority of patients had moderate or severe chronic damage on renal biopsy (Table 5.1).

Table 5.2. Independent risk factors associated with dialysis

Variables	**Estimated Hazard Ratio**	**95% Confidence Interval**	**p value**
dFLC ≥90% at 3 months	**0.24**	0.106 - 0.547	**0.001**
Log NT-proBNP	1.35	0.773 – 2.369	0.289
eGFR	0.97	0.878 – 1.068	0.564
Proteinuria	1.01	0.961 – 1.054	0.618

Forty-five of 84 patients from the whole cohort had renal amyloidosis in the absence of cardiac involvement and were defined as 'renal isolated.' Nine such patients were on RRT at baseline and 2 died before the 3 month evaluation. In light of our previous results, the remaining 34 'evaluable' patients were stratified using the 'percentage of baseline dFLC' method to ≥90% or <90% dFLC response within 3 months of baseline. Median renal survival among 11 patients who achieved a dFLC response of ≥90% within 3 months of baseline was 23.0 months (CI 7.3–undefined) compared to only 6.2 months (CI 3.0–12.5) among 23 patients who achieved lesser degrees of clonal response at the same timepoint (log rank test, p<0.007) (Figure 5.5), and 5.7 months (CI 1.9-undefined) among those who achieved a ≥90% dFLC response, but only after 12 months from baseline (log rank test, p<0.03). There was no significant difference in renal survival between those who achieved a dFLC response within 3 months of 50-89% compared to a dFLC

response of <50% (log rank test, p=0.83). By Cox regression analysis, the only independent factor associated with a requirement for RRT in the 45 patients with 'renal isolated' amyloidosis was achieving a dFLC response of ≥90% at 3 months (HR 0.62 [CI 0.057-0.655], p=0.008).

Figure 5.5. Renal survival calculated by Kaplan Meir analysis in evaluable patients with 'renal isolated' AL amyloidosis and eGFR <20ml/min/1.73 m^2 at presentation. Patients were stratified according to degree of clonal response at 3 months into dFLC response ≥90% and dFLC response <90% (p<0.007). Number at risk at certain timepoints is shown in panel below graph.

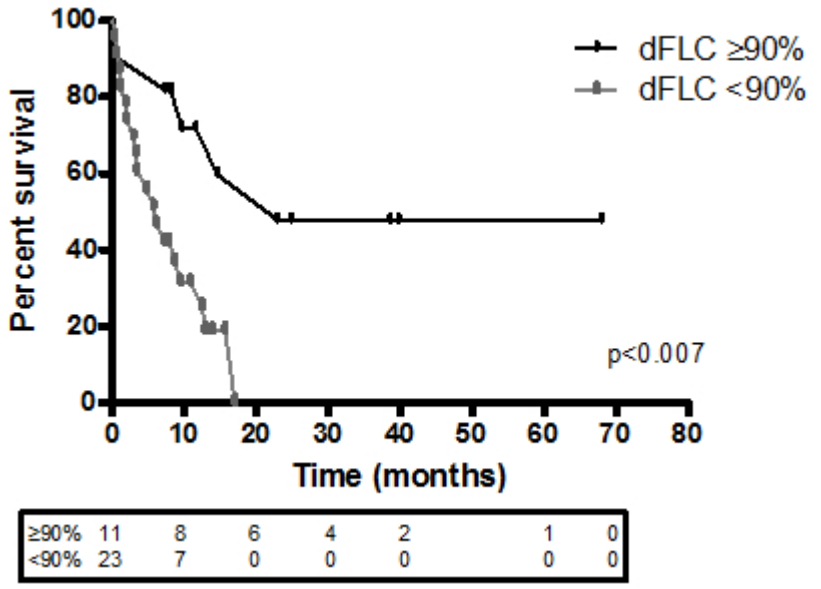


Figure 5.5

Time to composite endpoint of death or dialysis in relation to response to chemotherapy

Sixteen patients, who were dialysis dependent at baseline, were excluded from all analyses of time to the composite endpoint of death or dialysis. Twelve patients did not have an FLC assay measured at 3 months from baseline, in 10 cases due to death. Of the 10 patients who died, 4 died before receiving chemotherapy, 5 died during chemotherapy from progression of their systemic amyloidosis and 1 died from chemotherapy-related complications (sepsis). The remaining 56 patients were stratified according to dFLC response of <90% or ≥90% at 3 months. Among 18

patients who achieved a ≥90% dFLC response, median time to composite endpoint of death or dialysis was 17.3 months (CI 7.3-46.1) compared to 5.3 months (CI 3.4-7.6) among 38 patients who achieved a <90% response (log rank test, p<0.001) (Figure 5.6). The first event was death in 9 patients and dialysis in 27 patients. There was no significant difference in median time to death or dialysis between those patients who achieved a <50% dFLC response at 3 months and those who achieved a dFLC response of 50-89% (log rank test, p=0.53).

Figure 5.6 Time to composite endpoint of death or dialysis calculated by Kaplan Meier analysis in all evaluable patients with renal AL amyloidosis and eGFR <20 ml/min/1.73 m^2 at presentation, stratified according to degree of clonal response at 3 months into dFLC response ≥90% and dFLC response <90% (p<0.001). Number at risk at certain timepoints is shown in panel below graph.

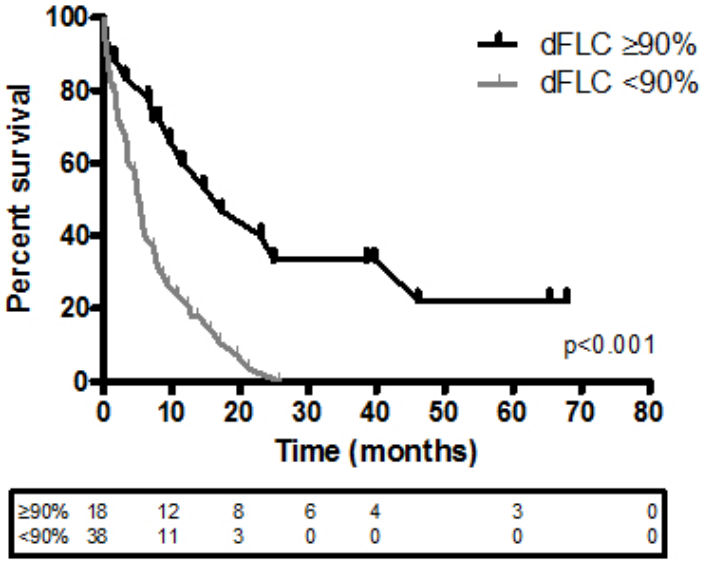


Figure 5.6

By Cox regression analysis, independent factors significantly associated with the composite endpoint of death or dialysis among all 68 patients who were dialysis independent at baseline were elevated NT-proBNP at presentation (HR 2.40 [CI 1.293-4.463], p=0.006) and achieving a dFLC response ≥90% at 3 months (HR 0.23 [CI 0.102-

0.505], p<0.001). Percentage dFLC response was also highly significant when incorporated as a continuous variable (HR 0.981 [CI 0.971-0.992], p=0.001)(Table 5.3). Neither presenting eGFR, nor proteinuria at presentation were significant predictors of the composite endpoint. Due to the high clonal response rates observed with bortezomib, a multivariable model in which dFLC response at 3 months of <90% or ≥90% was replaced by bortezomib vs no bortezomib was undertaken. The only factor independently associated with the same composite endpoint in this model was serum NT-proBNP concentration at presentation (HR 2.48 [CI 1.350-4.535], p=0.003) (Table 5.3).

Table 5.3. Independent risk factors associated with composite endpoint of death or dialysis

Variables	**Estimated Hazard Ratio**	**95% Confidence Interval**	**p value**
dFLC ≥90% 3 months	**0.23**	0.102 - 0.505	**<0.001**
Log NT-proBNP	**2.40**	1.293 – 4.463	**0.006**
eGFR	0.98	0.893 – 1.068	0.61
Proteinuria	1.01	0.962 – 1.064	0.66
Bortezomib vs No Bortezomib	0.57	0.317 – 1.011	0.054
Log NT-proBNP	**2.48**	1.350 – 4.535	**0.003**
eGFR	1.01	0.927 – 1.100	0.83
Proteinuria	1.02	0.966 – 1.070	0.53

Discussion

Response to chemotherapy is known to be one of the main determinants of patient survival in systemic AL amyloidosis.(81, 101) Two thirds of patients with systemic AL amyloidosis have renal involvement at diagnosis and renal outcome, as well as patient survival, is known to be influenced by response to chemotherapy.(130) However, no studies have been performed to specifically investigate whether the magnitude and speed of clonal response to chemotherapy in

patients who present with established advanced renal impairment influences time to requirement for RRT. Similarly, the merits of administering chemotherapy, which is invariably associated with substantial short-term morbidity, remain uncertain among AL amyloidosis patients who present with advanced CKD but do not have clinically significant extra-renal organ involvement by amyloid. Here we show for the first time, that the speed and magnitude of clonal response in patients presenting with a GFR of <20 ml/min/1.73 m^2 due to renal amyloidosis, directly influence the clinically important outcome measures of death, dialysis and the composite endpoint of death or dialysis, with markedly extended renal and patient survival among patients who achieved a clonal response of ≥90% within 3 months of baseline. Furthermore, we show that in patients with an eGFR of <20 ml/min/1.73 m^2 who do not have cardiac amyloidosis, chemotherapy can substantially delay the requirement for RRT. The findings presented here are analogous to the effect of chemotherapy in patients with advanced (Mayo stage 3) cardiac AL amyloidosis, (160) in which the speed and depth of clonal response directly influence patient survival. Until now it has not been clear whether the same degree and speed of clonal response can salvage renal function or if not, delay RRT in those who do not have cardiac involvement and whether patients with isolated renal amyloidosis require chemotherapy with the same degree of urgency as those with cardiac AL amyloidosis.

Importantly, this study does not prove beyond all doubt that aggressive chemotherapy aimed at achieving a rapid and deep clonal response delays dialysis and/or improves survival in this cohort of patients, since there was no prospective randomisation to a placebo arm or 'low intensity' chemotherapy arm. It does not therefore take into account those whose death or requirement for RRT may have been accelerated by chemotherapy. Nonetheless, the evidence for pursuing chemotherapy that is likely to achieve a rapid and deep clonal response in such patients is compelling; among the 47 patients in the whole cohort who died, 26 did so from progressive amyloidosis, including 4 patients who died before receiving chemotherapy; with only one death of the 26 deaths attributable to complications of chemotherapy. Similarly, there was no evidence of acute kidney injury complicating CKD among those in the cohort who received chemotherapy and only 2 patients out of the 78 who received chemotherapy required

discontinuation due to toxicity, one of whom was already receiving RRT at the time of commencement of chemotherapy, and the other of whom received a total of 8 cycles (first line thalidomide switched to bortezomib) to a complete clonal response and remains dialysis independent. Given that it would probably be considered unethical to withhold chemotherapy from patients with advanced renal dysfunction due to AL amyloidosis, particularly in light of the findings reported here, a prospective randomised trial to definitively answer this question will probably never be possible. Although bortezomib was associated with higher rates of rapid (within 3 months) and deep (≥90%) clonal response compared to non-bortezomib containing regimens, we were unable to demonstrate that administration of bortezomib was an independent predictor of outcome in this cohort. Nonetheless, we would encourage the use of bortezomib first line in patients with advanced renal impairment from AL amyloidosis due to the fact that it is generally well tolerated, no dose modification is necessary in patients with advanced renal impairment, and due to the speed and efficacy with which it can suppress the underlying clonal dyscrasia.

It is noteworthy that use of the established AL amyloidosis consensus criteria for measuring clonal response to chemotherapy, in which patients are required to have an absolute pre-treatment dFLC concentration of >50 mg/L to be evaluable, was associated with categorization of 12% patients as 'not evaluable', in accordance with the 15% patients reported in the consensus document.(159) However, it is interesting that, in this cohort of patients, the consensus criteria did not even predict patient survival, which is well known from larger studies of patients with AL amyloidosis to be associated with depth of hematologic response at 3 months.(161) However, the 'percentage of baseline dFLC' method used in our analyses, which has also been previously validated in AL amyloidosis,(130) predicted both patient and renal survival. Whilst the consensus criteria may be appropriate for determining eligibility of patients with AL amyloidosis for formal clinical trials, our 'real world' data indicates that the 'percentage of baseline dFLC' method is valid and applicable to all patients in a clinical practice setting, and may be superior in patients with established advanced CKD. This study is of insufficient size to recommend a change in the consensus criteria, but given the relative rarity of the disease, the need

for a true representation of clinical practice within clinical trials, and the established difficulties associated with enrolling sufficient numbers of patients with AL amyloidosis into most clinical trials, we believe that a specific comparison of these two methods among a large cohort of AL amyloidosis patients with established advanced CKD is warranted and, depending on the findings, may merit considering a change to the consensus criteria. In summary, chemotherapy should not be withheld from patients with advanced CKD due to renal AL amyloidosis. On the contrary, such patients should be treated urgently with the aim of achieving a rapid and deep clonal response, the result of which may be delayed dialysis and prolonged survival.

Chapter Six: Cardiorenal AL amyloidosis: risk stratification and outcomes based upon cardiac and renal biomarkers

Introduction

The amyloidoses are disorders of protein folding, in which a variety of proteins misfold and aggregate into fibrils that accumulate in tissues and disrupt organ function.(152) Immunoglobulin light chain (AL) amyloidosis is caused by deposition of fibrils derived from monoclonal immunoglobulin light chains and is the most common and serious type of systemic amyloidosis.(5) Renal and cardiac involvement respectively are present in approximately 70% and 50% of patients with systemic AL amyloidosis at diagnosis. Renal involvement manifests with nephrotic syndrome and progressive renal impairment,(101) and cardiac involvement with congestive cardiac failure. Progression to end-stage renal disease (ESRD) is one of the main determinants of morbidity in AL amyloidosis, whilst presence and severity of cardiac amyloidosis is the main determinant of mortality.(67, 68)

Cardiorenal syndrome is increasingly being recognised as a distinct clinical entity. Recent classifications have separated cardiorenal syndrome into 5 types; acute heart failure leading to acute kidney injury (Type 1), chronic abnormalities in cardiac function leading to progressive chronic kidney disease (CKD) (Type 2), acute deterioration in renal function leading to acute cardiac dysfunction (Type 3), chronic kidney disease contributing to progressive cardiac impairment (Type 4) and systemic diseases affecting both the heart and kidneys in both the acute and chronic setting (Type 5). Systemic AL amyloidosis is one of the main causes of type 5 cardiorenal syndrome.(162, 163)

The main established determinant of outcome in systemic AL amyloidosis is the depth of serum free light chain response to chemotherapy.(101, 155) Patients who achieve either a very good partial response (VGPR) or a complete response (CR) of the underlying hematologic condition have the highest chance of subsequent improvement in amyloidotic organ dysfunction, termed an 'organ' response.(101, 130, 154) In 2005, amyloidosis consensus criteria (ACC) were established to define individual organ involvement in systemic AL amyloidosis as well as individual organ responses.

In 2014, renal staging and 'early' renal response criteria (at 6 months) using eGFR and proteinuria to predict renal survival were developed for renal AL amyloidosis. Despite concurrent cardiac amyloidosis among 70% of the cohort that were employed to establish these criteria, alternative biomarkers such as N-terminal pro-b-type natriuretic peptide (NT-proBNP) were not included in the analysis.(154) Whilst early renal response was highly predictive of renal survival, it was not predictive of overall survival; furthermore, a composite endpoint including first of either death or dialysis was not explored. The current ACC response criteria have not therefore been specifically validated in patients with cardiorenal syndrome due to AL amyloidosis and have not been examined with respect to the key outcome measures in this population of death, dialysis or the composite endpoint of either death or dialysis.

We report renal and patient outcomes among 318 patients with systemic AL amyloidosis and cardiorenal syndrome who were prospectively enrolled into the UK AL Amyloidosis Chemotherapy (ALchemy) study between 2009 and 2016, in relation to their baseline clinical parameters and stratify them at different timepoints according to their amyloidotic organ function.

Patients and Methods

Patients

At the time of censor, 1000 patients with newly diagnosed AL amyloidosis had been enrolled into the prospective ALchemy study at the UK National Amyloidosis Centre (NAC). Cardiorenal syndrome was defined by the combination of more than 0.5g/24 hr of non-Bence Jones proteinuria and presence of cardiac involvement by echocardiography according to amyloidosis consensus criteria.(69) In rare cases in which echocardiography was suggestive but not conclusive with respect to presence of cardiac amyloidosis, it was supplemented by assessment of late gadolinium enhancement images on CMR, as previously defined.(157, 164) All analyses presented in this manuscript were performed in the cohort of patients with systemic AL amyloidosis who satisfied the above criteria for cardiorenal syndrome.

All patients underwent protocolized assessments at the NAC at baseline, 3, 6 and 12 months and subsequently at 6 monthly intervals, each comprising a complete clinical evaluation including volume status evaluation and medication review, as well as serum and urine biochemistry including assessment of renal and liver function, NT-proBNP, echocardiography, NYHA functional class, 6 minute walk test (6MWT), SAP scintigraphy,(65) and assessment of hematologic disease by serum free light chain (FLC) assay along with serum and urine immunofixation electrophoresis.

All patients were managed in accordance with the Declaration of Helsinki and provided written informed consent for study entry (REC reference 09/H0715/58) and publication of their data.

Assessment of Hematologic response

Details and doses of chemotherapy regimens were collected. All patients had serial FLC concentration prospectively monitored on blood samples obtained monthly during periods of chemotherapy treatment, and 1-3 monthly (depending on depth and stability of response) thereafter. Healthy polyclonal serum FLC concentration increases progressively through

advancing stages of chronic kidney disease (CKD)(122) which hinders the monitoring of monoclonal light chain disorders. In this study, the value of the FLC monoclonal component was estimated by subtracting the concentration of the uninvolved light chain from that of the amyloidogenic light chain to obtain the FLC difference (dFLC), a strategy previously validated in multiple myeloma and AL amyloidosis.(123, 130)

The FLC response to chemotherapy was determined according to previously validated ACC.(159) Since ACC define "evaluable" patients as those with a pre-treatment (baseline) dFLC of >50 mg/l, thus excluding 27/318 (9%) patients in this cohort, the percentage of the baseline dFLC that remained at the time of analysis (percentage method), also validated in AL amyloidosis,(130, 165) was additionally used to determine response to chemotherapy. A very good partial response (VGPR) was defined according to the ACC as an absolute dFLC of <40mg/L and by the percentage method as a ≥90% reduction of pre-treatment dFLC remaining after chemotherapy, as previously described.(130, 165)

Assessment of Organ Response

Organ responses were defined according to ACC;(91) cardiac progression as an increase in NT-proBNP of >30% and >300 ng/L and cardiac response as a reduction of NT-proBNP of >30% (and >300 ng/L decrease in patients with baseline NT-proBNP ≥ 650 ng/L). Renal progression was defined as a ≥ 25% reduction in eGFR and or a ≥ 50% increase in proteinuria and renal response was defined as a ≥ 50% reduction in proteinuria without a ≥ 25% reduction in eGFR. Additionally, 'early' renal progression was defined at six months from baseline as a ≥ 25% reduction in eGFR and 'early' renal response was defined at six months from baseline as a ≥ 30% reduction in proteinuria without a ≥ 25% reduction in eGFR, according to previously published amyloidosis consensus criteria.(154)

Patient and Renal Survival

Overall survival (OS) was defined as the time from baseline diagnostic evaluation at the NAC to patient death and was evaluated in all 318 patients. Renal survival was defined as the time from baseline diagnostic evaluation at the NAC to requirement for renal replacement therapy (RRT). For the analyses of renal survival, patients who were already established on RRT (n=7) at the time of their baseline evaluation were excluded, and those who died without requiring RRT were censored at the time of death. For analyses of time to the composite endpoint of death or dialysis, patients who were on RRT at baseline were excluded and an event was recorded as the first of either death or dialysis. Patient follow up was censored on 1st September 2016.

Statistical Analysis

Median patient survival, renal survival, and survival to the composite endpoint of 'dialysis or death', were determined by Kaplan-Meier (KM) survival analysis using GraphPad Prism v5.03 software. All analyses examining clonal or organ response at set time points were performed as landmark analyses. Univariable Cox regression was used to investigate the factors associated with the endpoints of death, dialysis, or the composite endpoint of 'dialysis or death' at baseline and at relevant time points for each of the three endpoints using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Multivariable Cox proportional hazards regression analysis was subsequently used to investigate factors independently associated with each of the three endpoints at the relevant time points. A significance level of 0.05 was used for all hypothesis tests.

Results

Baseline Characteristics and Patient Survival

Type 5 cardiorenal syndrome, defined by the combination of renal amyloidosis (more than 0.5g/24 hr of non-Bence Jones proteinuria by ACC organ involvement criteria) and cardiac

amyloidosis (>12 mm IVSd thickness on echocardiography without an alternative cause), was present in 318 patients at the time of diagnosis. Histological proof of amyloid was established in all patients including renal biopsy evidence of AL amyloid deposits in 150/318 (47%) cases and endomyocardial biopsy evidence of AL amyloid deposits in a further 6 patients; no patient in the whole cohort of 318 patients had either a cardiac or renal biopsy which failed to show amyloid. Among 162/318 cases who did not have endomyocardial or renal biopsy 'proof' of AL amyloid deposits, all had clear evidence of cardiac and renal amyloidosis on the basis of existing ACC accompanied by AL amyloid deposits in a biopsy specimen from an alternative site. In 310/318 patients, cardiac amyloidosis was definitively diagnosed by echocardiography (although frequently supplemented by characteristic CMR findings) but in the remaining 8 cases in which echocardiography was not definitively diagnostic of cardiac amyloidosis, its presence was confirmed by the finding of characteristic late gadolinium enhancement by CMR. Of the 168 patients who did not undergo a renal biopsy, 80/168 (47%) had nephrotic range proteinuria ($\geq$ 3g/24hr).

Baseline demographics and clinical characteristics of the whole cohort of 318 patients with cardiorenal syndrome are listed in Table 6.1. Median age at diagnosis was 66 years with M:F ratio of 1:1. Median eGFR was 55 ml/min/1.73 m^2 (range 10-100) with a median 24 hour urinary protein leak of 4.6 grams (range 0.5-26.8) and median serum albumin of 30 g/L (14-49). Mayo disease Stage was 1, 2 and 3 in 4 (1%), 92 (29%) and 222 (70%) of patients respectively. Median NT-proBNP was 5568 ng/L (range 145-70,295) with 66 (21%) patients having a concentration of >8500 ng/L (defined by amyloidosis staging criteria as Stage 3b disease).(68) Median follow up by reverse Kaplan-Meier (KM) analysis for the whole cohort was 47.5 months (range 0.1-83.5). A total of 199/318 (63%) patients died with a median overall survival of only 18.5 months by KM analysis. Among the 199 patients who died, median time to death was 5.0 months (range 0.1-54.8); 114/199 (57%) patients died within 6 months and 146/199 (73%) within 12 months of baseline.

Table 6.1 Baseline characteristics of 318 patients with systemic AL amyloidosis and cardiorenal syndrome

Demographic or clinical characteristic			**No. of Patients (N =318)**	**%**
Male sex	N		177	55
Female sex	N		141	45
Age (years)	Median		66	
	Range		39-89	
Patients presenting on RRT	N		7	2
eGFR (ml/min/1.73 m^2)	Median		55	
	Range		10-100	
CKD Stage				
1				18
2				26
3				32
4				16
5				8
Serum albumin (g/L)	Median		30	
	Range		14-49	
24hr urinary protein loss (g)	Median		4.6	
	Range		0.5-26.8	
NT-proBNP (ng/L)	Median		5568	
	Range		145-70,295	
Troponin T (ng/L)	Median		87	
	Range		<10-1870	
Mayo Stage				
1				1
2				29
3a				49
3b				21
Amyloidogenic light chain (n)	Lambda	N	260	82
	Kappa	N	58	18
λ sFLC concentration in AL (λ) patients (mg/L)	Median		253	
	Range		7-6500	
κ sFLC concentration in AL (κ) patients (mg/L)	Median		575	
	Range		10-10300	
Haemoglobin (g/dL)	Median		12.9	
	Range		8.3-18.1	
Serum creatinine (µmol/L)	Median		107	
	Range		26-979	
Bilirubin (µmol/L)	Median		6	
	Range		1-70	
Alkaline Phosphatase (u/L)	Median		98	
	Range		35-2142	
Supine systolic blood pressure (mmHg)	Median		112	
	Range		78-194	
Standing systolic blood pressure (mmHg)	Median		108	
	Range		63-192	
6 minute walk test (6MWT) metres	Median		276	
	Range		0-578	
NYHA class	0-2		236	
	3-4		82	
Amyloid load by SAP scintigraphy (n=312)	None	N	50	15
	Small	N	102	33
	Moderate	N	86	28

	Large	N	74	24
Left ventricular posterior wall thickness end diastole (LVPWd) (mm)	Median Range		14 11-24	
E/E' Prime	Median Range		14 4.5-48	
Lateral S Wave (m/s)	Median Range		0.07 0.02-0.17	
Left Ventricular Ejection Fraction (LVEF) (%)	Median Range		56 23-80	

Fifty (16%) patients in the whole cohort required RRT during follow up (of whom 7 were already dialysis dependent at baseline). Median time to commencement of dialysis among those 50 patients was 6.6 months (range 0-57.8). Twenty-four patients commenced dialysis within 6 months and 31 within 12 months of baseline. Median overall survival from diagnosis in the cohort of 50 patients who required RRT by KM analysis was 37.4 months, and median survival from commencement of RRT in those 50 patients was 23.0 months.

Chemotherapy

Chemotherapy was planned in all 318 patients but was not delivered in 23 (7%) cases, all of whom died prior to starting treatment (median time to death in these 23 patients was 26 days). Among those 295 patients who did receive chemotherapy, 156 (53%) received a bortezomib-based regimen first line, 104 (35%) received a thalidomide-based regimen first line and 35 (12%) received a first line regimen containing neither bortezomib nor thalidomide. Only one patient received upfront high dose melphalan conditioning with autologous stem cell transplantation (ASCT). Median number of chemotherapy cycles administered first line was 4 (1-9).

Disease-related Predictors of Outcome at Baseline

Univariable analysis of baseline parameters associated with death, dialysis, and the composite endpoint of death or dialysis are listed in Table 6.2a as continuous and categorical variables. Baseline parameters that were significantly associated with death were NT-proBNP concentration

(p<0.001), Mayo Stage (p<0.001), eGFR (p<0.001), supine systolic blood pressure (p<0.001), 6 minute walk test (6MWT) distance (p<0.001), NYHA class (p<0.001) and ECOG performance status (p<0.001). Baseline parameters that were significantly associated with dialysis were NT-proBNP (p<0.001), Mayo Stage (p<0.001) and eGFR (p<0.001). Degree of proteinuria alone was not significantly associated with risk of dialysis, although 'Renal Stage' (eGFR <50ml/min/1.73m^2 and/or >5g/24hr of proteinuria)(154) was (Stage 3 vs Stage 1; HR 9.40 [3.11-28.37], p<0.001) (Table 6.2a).

Multivariable analyses including baseline eGFR, NT-proBNP and supine systolic BP as categorical variables revealed NT-proBNP >8500ng/L to be a significant predictor of death (HR 3.01 [2.22-4.10], p<0.001), dialysis (HR 1.98 [1.01-3.88], p=0.045) and the composite endpoint (HR 2.91 [2.16-3.91], p<0.001). Supine systolic BP <100mmHg was an independent predictor of death (HR 1.70 [1.23-2.35], p=0.001) and the composite endpoint (HR 1.45 [1.06-1.99], p=0.021), whereas eGFR <30ml/min/1.73 m^2 was an independent predictor of dialysis (HR 4.86 [2.55-9.25], p<0.001) and the composite endpoint (HR 1.73 [1.26-2.39], p=0.001) (Table 6.2b).

Table 6.2a. Univariable analysis of disease-related predictors of death, dialysis and the composite endpoint at the time of diagnosis

Variable		Death		Dialysis		Death or Dialysis	
		HR (CI)	P value	HR (CI)	P value	HR (CI)	P value
Supine systolic BP							
Continuous		0.98 (0.97-0.99)	***<0.001***	1.02 (1.00-1.03)	***0.036***	0.99 (0.98-1.00)	***0.006***
Categorical	≥ 100mmHg	1		1		1	
	< 100mmHg	1.84 (1.35-2.50)	***<0.001***	0.31 (0.10-1.02)	0.050	1.52 (1.12-2.05)	***0.007***
24 hour proteinuria							
Continuous		0.97 (0.93-0.99)	0.050	1.07 (1.01-1.12)	***0.013***	0.98 (0.95-1.01)	0.212
Categorical	0-3g/24h	1		1		1	
	3-5g/24h	1.40 (1.0-1.9)	***0.040***	0.50 (0.23-1.10)	0.084	1.29 (0.95-1.76)	0.101
	> 5g/24h	1.00 (0.7-1.5)	0.990	0.68 (0.31-1.51)	0.344	0.91 (0.63-1.33)	0.638
Serum albumin							
Continuous		1.00 (0.98-1.10)	0.960	1.01 (0.97-1.05)	0.690	1.01 (0.99-1.02)	0.455
Categorical	> 30g/L	1		1		1	
	< 30g/L	1.07 (0.85-1.50)	0.390	1.16 (0.64-2.11)	0.615	1.27 (0.96-1.66)	0.089
Renal Stage							
Categorical							
	Stage 1	1		1		1	
	Stage 2	0.93 (0.67-1.30)	0.675	3.55 (1.23-10.29)	***0.019***	1.15 (0.83-1.59)	0.399
	Stage 3	1.28 (0.85-1.92)	0.232	9.40 (3.11-28.37)	***<0.001***	1.73 (1.15-2.58)	***0.007***
eGFR							
Continuous		0.99 (0.99-0.99)	***<0.001***	0.97 (0.96-0.98)	***<0.001***	0.99 (0.98-0.99)	***<0.001***
Categorical	CKD 1	1		1		1	
	CKD 2	1.08 (0.68-1.72)	0.730	0.55 (0.17-1.80)	0.321	0.98 (0.63-1.53)	0.938
	CKD 3a	1.50 (0.94-2.40)	0.086	1.10 (0.35-2.40)	0.876	1.36 (0.87-2.14)	0.179
	CKD 3b	1.31 (0.76-2.31)	0.331	1.61 (0.49-5.29)	0.431	1.39 (0.83-2.34)	0.215
	CKD 4	1.89 (1.16-3.08)	***0.011***	6.37 (2.40-16.88)	***<0.001***	2.65 (1.67-4.21)	***<0.001***
	CKD 5	1.83 (1.06-3.18)	***0.031***	7.33 (2.53-21.29)	***<0.001***	3.26 (1.86-5.71)	***<0.001***
NT-proBNP							
Continuous		1.00 (1.00-1.00)	***<0.001***	1.00 (1.00-1.00)	***<0.001***	1.00 (1.00-1.00)	***<0.001***
Categorical	≤ 8500ng/L	1		1		1	
> 8500ng/L		3.30 (2.50-4.42)	***<0.001***	3.00 (1.70-5.40)	***<0.001***	3.50 (2.62-4.61)	***<0.001***
Mayo Stage							
Categorical							
	Stage 2	1		1		1	
	Stage 3a	2.47 (1.69-3.61)	***<0.001***	1.17 (0.56-2.44)	0.673	2.29 (1.61-3.27)	***<0.001***
	Stage 3b	3.64 (2.39-5.53)	***<0.001***	4.14 (1.92-8.95)	***<0.001***	4.17 (2.78-6.25)	***<0.001***
NYHA Class							
Categorical	Class 1	1		1		1	
	Class 2	1.86 (1.07-3.21)	***0.027***	2.64 (0.79-8.79)	0.114	2.16 (1.25-3.72)	***0.006***
	Class 3	4.71 (2.65-8.35)	***<0.001***	2.48 (0.59-10.49)	0.216	5.07 (2.86-8.99)	***<0.001***
	Class 4	18.6 (6.01-57.70)	***<0.001***	0.00 (0.00-undefined)	0.987	17.44 (5.65-53.84)	***<0.001***

ECOG Performance Status							
Categorical	Class 0	1		1		1	
	Class 1	1.60 (0.73-3.50)	0.240	1.61 (0.49-5.36)	0.435	1.46 (0.73-2.93)	0.282
	Class 2	4.10 (1.90-8.83)	***<0.001***	1.85 (0.53-6.43)	0.332	3.29 (1.64-6.52)	***0.001***
	Class 3&4	6.10 (2.60-14.0)	***<0.001***	1.30 (0.22-7.91)	0.770	4.62 (2.17-9.86)	***<0.001***
6 minute walk test (6MWT) distance							
Continuous		0.99 (0.99-0.99)	***<0.001***	1.00 (0.99-1.02)	0.496	0.99 (0.99-0.99)	***<0.001***
Categorical							
	<300 metres	1					
	≥300 metres	0.26 (0.16-0.41)	***<0.001***	0.68 (0.22-2.20)	0.530	0.31 (0.20-0.48)	***<0.001***
Echocardiographic parameters							
	LVSd mm	1.06 (0.92-1.23)	0.416	0.90 (0.64-1.27)	0.555	1.07 (0.93-1.24)	0.300
	LVPW mm	0.99 (0.85-1.15)	0.905	1.20 (0.84-1.71)	0.315	0.98 (0.85-1.13)	0.755
	E/E’	1.03 (1.01-1.04)	***0.001***	0.97 (0.93-1.02)	0.347	1.02 (1.01-1.04)	***0.012***
	LVEF %	0.97 (0.95-0.98)	***<0.001***	1.01 (0.98-1.05)	0.334	0.97 (0.96-0.99)	***<0.001***

Table 6.2b. Multivariable analysis of disease-related predictors of death, dialysis and the composite endpoint at diagnosis, 6 months and 12 months

Multivariable	**Death**		**Dialysis**		**Death or Dialysis**	
	HR (CI)	***P value***	***HR (CI)***	***P value***	***HR (CI)***	***P value***
At diagnosis						
NT-proBNP >8500ng/L	3.01 (2.22-4.10)	***<0.001***	1.98 (1.01-3.88)	***0.045***	2.91 (2.16-3.91)	***<0.001***
eGFR < 30ml/min	1.14 (0.81-1.60)	0.445	4.86 (2.55-9.25)	***<0.001***	1.73 (1.26-2.39)	***0.001***
Supine systolic BP <100mmHg	1.70 (1.23-2.35)	***0.001***	0.39 (0.12-1.27)	0.117	1.45 (1.06-1.99)	***0.021***
Change at 6 months from baseline						
Increase NT-proBNP >30%	2.17 (1.22-3.88)	***0.009***	1.55 (0.77-3.10)	0.215	1.70 (1.03-2.81)	***0.037***
Reduction eGFR ≥ 25%	1.54 (0.90-2.65)	0.120	3.07 (1.51-6.26)	***0.002***	1.95 (1.20-3.18)	***0.007***
dFLC response ≥ 90%	0.42 (0.24-0.73)	***0.002***	0.54 (0.28-1.07)	0.078	0.42 (0.26-0.69)	***0.001***
Change at 12 months from baseline						
Increase NT-proBNP >30%	3.67 (1.79-7.53)	***<0.001***	2.85 (1.29-6.32)	***0.010***	3.05 (1.68-5.52)	***<0.001***
Reduction eGFR ≥ 25%	1.23 (0.63-2.42)	0.546	3.04 (1.28-7.25)	***0.012***	1.70 (0.96-3.05)	0.070
dFLC response ≥ 90%	0.50 (0.24-1.02)	0.060	0.62 (0.29-1.34)	0.230	0.58 (0.33-1.04)	0.068

Table 6.2c. Univariable analysis of hematologic responses at 3, 6 and 12 months as predictors of death, dialysis and the composite endpoint

Variable		**Response Rate %**	**Death**		**Dialysis**		**Death or Dialysis**	
			HR (CI)	***P value***	***HR (CI)***	***P value***	***HR (CI)***	***P value***
dFLC <40mg/L at 3 months								
Categorical	N		1		1		1	
	Y		0.28 (0.17-0.46)	***<0.001***	0.31 (0.14-0.70)	***0.005***	0.32 (0.20-0.50)	***<0.001***
% dFLC response at 3 months								
Continuous			0.98 (0.97-0.99)	***<0.001***	0.99 (0.98-1.00)	***0.018***	0.98 (0.98-0.99)	***<0.001***
Categorical	< 50%	28	1		1		1	
	50-89%	33	0.43 (0.27-0.68)	***<0.001***	0.63 (0.28-1.43)	0.274	0.46 (0.29-0.72)	***0.001***
	≥ 90%	39	0.17 (0.10-0.29)	***<0.001***	0.24 (0.10-0.61)	***0.003***	0.20 (0.12-0.33)	***<0.001***
dFLC <40mg/L at 6 months								
Categorical	N		1		1		1	
	Y		0.22 (0.13-0.36)	***<0.001***	0.32 (0.13-0.75)	***0.009***	0.23 (0.14-0.40)	***<0.001***
% dFLC response at 6 months								
Continuous			0.98 (0.97-0.99)	***<0.001***	0.99 (0.97-1.00)	***0.004***	0.98 (0.97-0.99)	***<0.001***
Categorical	< 50%	15	1		1		1	
	50-89%	31	0.41 (0.23-0.73)	***0.003***	0.69 (0.28-1.73)	0.965	0.51 (0.27-0.84)	***0.027***
	≥ 90%	54	0.18 (0.10-0.33)	***<0.001***	0.24 (0.09-0.64)	***0.004***	0.17 (0.09-0.33)	***<0.001***
dFLC <40mg/L at 12 months								
Categorical	N		1		1		1	
	Y		0.39 (0.20-0.76)	***0.006***	0.47 (0.17-1.34)	0.161	0.47 (0.23-0.96)	***0.039***
% dFLC response at 12 months								
Continuous			0.98 (0.97-0.99)	***<0.001***	0.98 (0.97-0.99)	0.053	0.98 (0.97-0.99)	***<0.003***
Categorical	< 50%	11	1		1		1	
	50-89%	28	0.42 (0.19-0.92)	***0.030***	0.48 (0.12-1.89)	0.297	0.77 (0.28-2.09)	0.610
	≥ 90%	61	0.15 (0.06-0.33)	***<0.001***	0.20 (0.05-0.78)	***0.021***	0.31 (0.11-0.84)	***0.022***

Outcome in Relation to Hematologic Responses at 3, 6 and 12 months from Baseline

Univariable analyses of outcome in relation to hematologic response to chemotherapy using both the ACC dFLC method (dFLC <40mg/L) and 'percentage' dFLC method are listed in Table 6.2c. As expected, depth of clonal response was a highly significant predictor of all three outcome measures. Specifically, achieving a dFLC of ≥ 90% at 3, 6 and 12 months was a highly significant (protective) predictor of death (HR 0.15 [0.06-0.33], p<0.001), dialysis (HR 0.20 [0.05-0.78], p=0.021) and the composite endpoint (HR 0.31, [0.11-0.84], p=0.022), compared to lesser degrees of clonal response.

Outcomes in Relation to Change in Organ Function at 6 months from Baseline

In light of the 2014 'early' amyloidosis renal response criteria,(154) we assessed renal response at 6 months from baseline and its influence on death, dialysis and the composite endpoint. Univariable analyses revealed that early renal progression (reduction in eGFR of ≥ 25%) was predictive of death (HR 1.80 [1.07-3.05], p=0.028), dialysis (HR 3.45 [1.44-8.23], p=0.005) and the composite endpoint of death and dialysis (HR 1.96 [1.16-3.32], p=0.012). Interestingly however, 'early' renal response (≥ 30% reduction in proteinuria without a ≥ 25% reduction in eGFR) was not significantly associated with death (HR 0.76 [0.41-1.42], p=0.389), dialysis (HR 0.69 [0.25-1.93], p=0.490) or the composite endpoint (HR 0.80 [0.49-1.47], p=0.478).

In view of the univariable findings, multivariable analyses were performed with increase in NT-proBNP >30%, reduction in eGFR of ≥ 25%, and dFLC response of ≥90% at 6 months from baseline (Table 6.2b), as categorical variables. Increase in NT-proBNP >30% was predictive of death (HR 2.17 [1.22-3.88], p=0.009) and the composite endpoint (HR 1.70 [1.03-2.81], p=0.037) but not dialysis. Reduction in eGFR of ≥ 25% was highly predictive of dialysis (HR 3.017 [1.51-6.26], p=0.002) and the composite endpoint (HR 1.95 [1.20-3.18], p=0.007) but not death, and dFLC response of ≥90% was (protectively) predictive of death (HR 0.42 [0.24-0.73], p=0.002) and the composite endpoint (HR 0.42 [0.26-0.69], p=0.001) but not dialysis.

Outcomes in Relation to Change in Organ Function at 12 months from Baseline

Univariable analyses using the ACC for renal organ response at 12 months were performed. Renal progression (≥ 25% reduction in eGFR and/or a ≥ 50% increase in proteinuria) was not significantly associated with death (HR 1.63 [0.86-3.08], p=0.127) or the composite endpoint (HR 1.70 [0.91-3.20], p=0.096) but was significantly associated with dialysis (HR 3.29 [1.17-9.24], p=0.024). Renal response (≥ 50% reduction in proteinuria without a ≥ 25% reduction in eGFR) was not significantly associated with death (HR 0.96 [0.43-2.14], p=0.925), dialysis (HR 0.58 [0.16-2.06], p=0.406) or the composite endpoint (HR 0.98 [0.47-2.02], p=0.947). Univariable analyses using the ACC for cardiac organ response at 12 months were performed. Cardiac progression (increase in NT-proBNP of >30%) was a highly significant predictor of death (HR 5.32 [2.78-10.16], p<0.001) and the composite endpoint (HR 3.10 [1.61-5.96], p<0.001) but not dialysis, although demonstrated a trend to significance (HR 2.49 [0.91-6.78], p=0.075). Cardiac response (reduction in NT-proBNP of >30%) was a highly significant (protective) predictor of death (HR 0.24 [0.12-0.51], p<0.001) and the composite endpoint (HR 0.42 [0.22-0.80], p=0.009) but not dialysis (HR 0.54 [0.20-1.42], p=0.214).

Multivariable analyses for predictors of death, dialysis and the composite endpoint were performed including increase in NT-proBNP >30%, reduction in eGFR of ≥ 25%, and dFLC response of ≥90% at 12 months from baseline as categorical variables (Table 6.2b). An increase in NT-proBNP of >30% was a highly significant predictor of all three outcomes; death (HR 3.67 [1.79-7.53], p<0.001), dialysis (HR 2.85 [1.29-6.32], p=0.010) and the composite endpoint (3.05 [1.68-5.52], p<0.0001). Reduction in eGFR of ≥ 25% was not significantly associated with death (HR 1.23 [0.63-2.42], p=0.546) or the composite endpoint (HR 1.70 [0.96-3.05], p=0.070) but was associated with dialysis (HR 3.04 [1.28-7.25], p=0.012) and dFLC response of ≥90% was not significantly associated with any of the three outcomes.

Discussion

Systemic AL amyloidosis is a multisystem disease with a heterogeneous clinical presentation and clinical course, as well as a widely varying prognosis. Symptoms, quality of life and patient survival are dependent upon the presence and severity of individual organ involvement and the hematologic response to chemotherapy, the mainstay of treatment for the disease. International amyloidosis consensus criteria (ACC) exist to define individual organ involvement by amyloid at the time of diagnosis as well as to define response of individual organs to chemotherapy. It is well established that presence and severity of cardiac amyloidosis at diagnosis is the main determinant of patient survival in AL amyloidosis,(68) whilst presence and severity of renal dysfunction from amyloid determines the risk of progression to end stage renal disease (ESRD), a major determinant of quality of life. Systemic AL amyloidosis is a cause of cardiorenal syndrome, but there are no studies that have specifically looked at patient and renal survival in patients with systemic AL amyloidosis presenting with combined cardiac and renal amyloidotic dysfunction. Here we show for the first time that almost one third of patients diagnosed with systemic AL amyloidosis present with Type 5 cardiorenal syndrome, and that their outcomes are poor despite chemotherapy. Nearly two thirds of patients in the cardiorenal cohort died, among whom median time to death was only 5 months; median overall survival in this cohort by Kaplan Meier analysis was only ~18 months. In addition, 16% patients developed ESRD, the majority of whom reached dialysis within 12 months of diagnosis and median (range) time to the composite endpoint of either death or dialysis by KM analysis in the whole cohort was only 9.3 (5.5-13.1) months.

The data presented here are novel in that they concern three hard outcome measures, patient survival, dialysis, and the composite of either death or dialysis in the one third of patients with AL amyloidosis who present specifically with cardiorenal syndrome. At the time of diagnosis, the three disease-related parameters that were highly significant independent predictors of all three outcomes were NT-proBNP concentration, Mayo Stage and eGFR. As previously and

extensively reported,(101, 130, 155) the clonal response to chemotherapy within 12 months of diagnosis was also predictive of both patient and renal survival.

Interestingly, an increase or reduction in proteinuria at 12 months, both of which are included in the ACC renal response criteria, did not predict death, dialysis, or the composite endpoint. Additionally, an 'early' renal response (at 6 months from baseline) was not significantly associated with any of the three outcome measures. A reduction of eGFR of ≥ 25% at 6 months was predictive of dialysis and the composite endpoint in keeping with previous findings by Palladini and colleagues,(154) whilst an increase in NT-proBNP of >30% at 12 months was predictive of all three outcome measures. This highlights for the first time the strong association between both baseline NT-proBNP concentration, its increase at 12 months (ACC cardiac progression) and each of the three hard outcome measures of death, dialysis and the composite endpoint. Furthermore, whilst renal stage (eGFR <50ml/min and proteinuria of >5g/24hr) at baseline was highly predictive of renal survival alone; proteinuria, both at baseline and its change at 6 and 12 months was not predictive of any of the three outcomes in this cohort of patients, possibly due to the high early 'event' rate for both death and dialysis.

An increase or reduction in proteinuria in the absence of worsening GFR has previously been shown to be predictive of renal survival in patients with isolated renal AL amyloidosis.(130) The pathophysiology of reduction in proteinuria in AL amyloidosis is thought to relate to amyloid regression, or perhaps absence of ongoing amyloid accumulation, which is dependent upon adequate suppression of the underlying clonal disorder.(166) However, in clinical practice, a significant improvement in proteinuria rarely occurs before six months to one year; it may be therefore, that in this distinct cohort of patients with cardiorenal AL amyloidosis, the high early death, and to a lesser degree dialysis rates, preclude the use of proteinuria as a prognostic biomarker within the first year of diagnosis.

Based upon our study, we aimed to risk stratify patients with cardiorenal syndrome; at baseline, based upon eGFR and NT-proBNP (Table 6.3) and thereafter, based upon change in biomarkers within 12 months from diagnosis (Figure 6.1). At baseline, low risk was defined as

an NT-proBNP of ≤8500ng/L and eGFR ≥ 30 ml/min/1.73 m^2, intermediate risk as either NT-proBNP of >8500ng/L or eGFR <30 ml/min/1.73 m^2 and high risk as NT-proBNP of >8500ng/L and eGFR <30 ml/min/1.73 m^2 at baseline. High risk patients had a substantially increased risk of death (HR 3.12 [2.18-4.64], p<0.001), dialysis (HR 10.34 [4.66-22.91], p<0.001) and the composite endpoint (HR 4.76 [3.24-7.00], p<0.001) (Table 3). In all patients with systemic AL amyloidosis and cardiorenal syndrome, the outcome of dialysis was predominantly dictated by ≥ 25% reduction in eGFR at 6 months, but at 12 months an increase in NT-proBNP of >30% was the most significant predictor of death, dialysis and the composite endpoint (Figure 6.1).

Table 6.3 Risk stratification of patients with systemic AL amyloidosis and cardiorenal syndrome based upon baseline NT-proBNP and eGFR

Variable	**Death**		**Dialysis**		**Death or Dialysis**	
	HR (CI)	**P value**	**HR (CI)**	**P value**	**HR (CI)**	**P value**
Cardiorenal Risk Stage						
Low risk	1		1		1	
Intermediate risk	2.66 (1.93-3.66)	***<0.001***	2.82 (1.37-5.77)	***0.005***	2.89 (2.12-3.92)	***<0.001***
High risk	3.12 (2.18-4.64)	***<0.001***	10.34 (4.66-22.91)	***<0.001***	4.76 (3.24-7.00)	***<0.001***

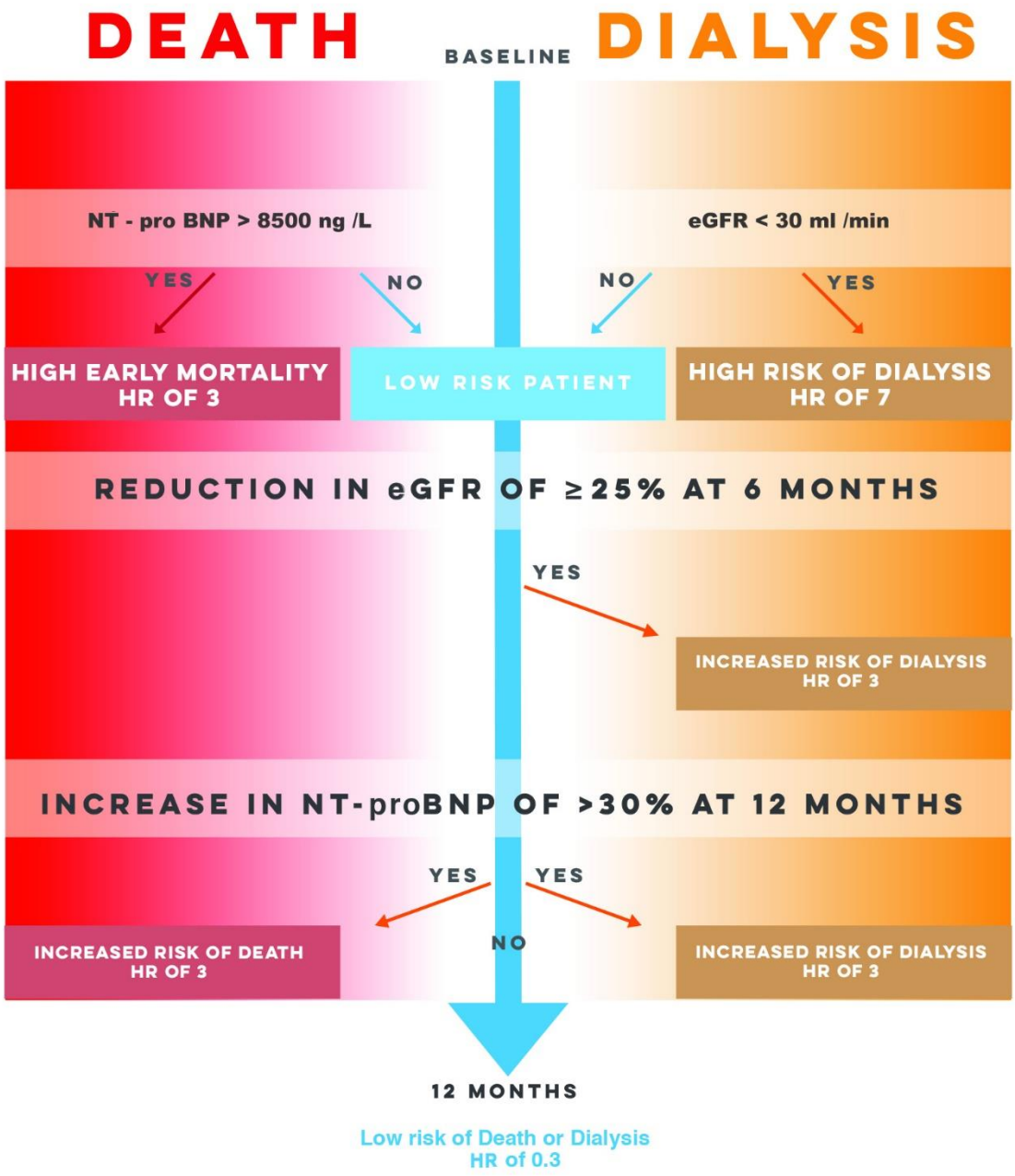
Limitations of our study include the relatively low incidence of dialysis in this cohort of patients. A total of 50 patients required dialysis of whom 50% were on dialysis by 6 months. Hemodialysis is the acute RRT therapy of choice in the UK and it may be that a small number of patients were deemed unsuitable to receive hemodialysis due to concurrent advanced cardiac amyloidosis, autonomic nerve dysfunction and associated hypotension, as well as patient choice. Due to the nature of our referral centre, these details were not available to us. Nonetheless, the authors feel that this 'real world' data regarding the specific cohort of patients with systemic AL amyloidosis who have type 5 cardiorenal syndrome is key in guiding clinicians who counsel such patients about their treatment and outcomes within the first year of diagnosis.

In summary, approximately one third of patients with systemic AL amyloidosis have cardiorenal syndrome at the time of diagnosis. Despite the complex relationship between NT-proBNP, renal excretory function, and cardiac function, our data indicates that hard outcome

measures in patients with systemic AL amyloidosis and type 5 cardiorenal syndrome are predominantly dictated by both baseline NT-proBNP and its change at 12 months. The key renal biomarker in this cohort is eGFR and not proteinuria. The initial aim of therapy, be it chemotherapy to prevent ongoing accumulation of amyloid and progressive amyloidotic organ dysfunction or symptomatic management of fluid balance, should be to prevent loss of ≥ 25% of baseline eGFR within 6 months and an increase in NT-proBNP of >30% within 12 months of diagnosis. Our data suggest that, irrespective of changes in proteinuria, and to some degree, GFR, changes in NT-proBNP (ACC cardiac progression) may be the most important independent predictor of death or a requirement for renal replacement therapy in such patients. Further work, possibly including novel methods of tracking end organ response, is needed to refine the current amyloid consensus criteria in order to reflect the needs of patients with multi-system disease from AL amyloidosis.

Figure 6.1

Risk stratification of patients with systemic AL amyloidosis and cardiorenal syndrome based upon NT-proBNP and eGFR at different timepoints within the course of the first year after diagnosis.



Chapter Seven: Bioimpedance vector analysis for the detection of extra-cellular volume overload, a new prognostic biomarker, coupled with sarcopenia in systemic AL amyloidosis

Introduction

The amyloidoses are disorders of protein folding, in which a variety of proteins misfold and aggregate into fibrils that accumulate in tissues and disrupt organ function.(152) Immunoglobulin light chain (AL) amyloidosis is caused by deposition of fibrils derived from monoclonal immunoglobulin light chains and is the most common and serious type of systemic amyloidosis.(5) Renal and cardiac involvement are present in approximately 70% and 50% of patients with systemic AL amyloidosis at diagnosis respectively. Renal involvement manifests with nephrotic syndrome and progressive renal impairment(101) and cardiac involvement with elevated plasma N-terminal pro-b-type natriuretic peptide (NT-proBNP) concentration and congestive cardiac failure such that both extracellular volume overload and sarcopenia are common.

Body composition assessment using bioimpedance vector analysis (BIVA) is a non-invasive tool that has been validated in patients with both chronic kidney disease and cardiovascular disease.(90, 167) It provides information on diagnosis,(168) volume status,(169) cachexia,(170) and overall prognosis(170) in patients with chronic diseases, including those with heart failure.(171) Abnormalities in volume status are common in cardiac amyloidosis and nutritional status at the time of diagnosis has been shown to influence prognosis in systemic AL amyloidosis.(172) and nutritional counselling has been shown to preserve body weight and

quality of life.(173) Nonetheless, body composition assessment via BIVA has not previously been described in patients with systemic AL amyloidosis.

We undertook a pilot study of BIVA to evaluate body composition in 300 patients at the time of diagnosis of systemic AL amyloidosis who were prospectively enrolled into the UK AL amyloidosis chemotherapy (ALchemy) study.

Patients and Methods

Patients

All patients who attended the National Amyloidosis Centre (NAC) with newly diagnosed systemic AL amyloidosis between April 2016 to April 2017 and were enrolled into ALchemy, a prospective observational AL Chemotherapy study, underwent body composition assessment via BIVA using InBody 770 in conjunction with routine clinical, biochemical, echocardiographic and scintigraphic assessments.(130)

Renal involvement was defined as non-Bence Jones proteinuria of more than 0.5g/24 hr according to the amyloidosis international consensus criteria,(69) and cardiac involvement was defined by echocardiography according to international consensus criteria.(69) In cases in which there was uncertainty regarding involvement of the heart by amyloid on the basis of echocardiography, additional cardiac magnetic resonance imaging was performed and cardiac involvement was defined on the basis of native T1 and/or extracellular volume measurement, as previously reported.(156, 157)

All patients underwent protocolized assessments every 3-6 months at the NAC, each assessment comprising clinical evaluation, serum and urine biochemistry including assessment of renal and liver function, NT-proBNP, echocardiography, SAP scintigraphy,(65) and assessment of hematological disease by serum free light chain (FLC) assay, and serum and urine immunofixation electrophoresis.

All patients were managed in accordance with the Declaration of Helsinki and provided written informed consent for study entry (REC reference 09/H0715/58) and publication of their data.

Bioelectrical impedance vector analysis (BIA)

Single frequency BVIA was performed, as previously described.(174) Extracellular water (ECW), total body water (TBW), fat-free mass (FFM), body fat mass (BFM), percentage body fat (%BF) and skeletal muscle mass (SMM) were calculated according to the manufacturer's software (InBody 770, Derwent Health Care, Seoul, South Korea). In order to confirm repeatability of BIA measurements in this patient population, 28 patients with systemic amyloidosis underwent serial BIA measurements across 3 consecutive days. Lin's concordance correlation coefficient was calculated and revealed good reproducibility for all four measurements of weight, SMM, %BF and ECW/TBW ratio with Rho_C of 0.999, 0.996, 0.992 and 0.986 respectively.

The presence of extracellular volume overload was defined according to ECW/TBW ratio.(175) Patients were categorised into 3 grades of ECV state; normal (0.360-0.390), mild/moderate overload (0.390-0.410), and severe overload (>0.410).

Sarcopenia was defined as Skeletal Mass Index (SMI) calculated as skeletal muscle/height^2 (kg/m^2) where skeletal muscle was estimated using BIA. For men, moderate sarcopenia was SMI between 8.51 and 10.75 kg/m^2 and severe sarcopenia was SMI < 8.50 kg/m^2. For women moderate sarcopenia was SMI between 5.76 and 6.75 kg/m^2 and severe sarcopenia was SMI < 5.75 kg/m^2, as previously described.(176, 177)

Chemotherapy

Chemotherapy was undertaken as per UK amyloidosis guidelines.(178) Twenty-three of 300 (6%) patients died prior to receiving systemic chemotherapy, 238/277 (86%) patients received a

first line bortezomib regimen, 5/277 (1%) a first line thalidomide regimen and 34//277 (13%) received first line chemotherapy containing neither thalidomide nor bortezomib. Median number of cycles administered first line was 3 (Range 1-7).

Statistical Analysis

Correlation was performed using Pearson correlation coefficient. Means within subgroups were compared by one way Anova tests. Analyses were performed using Graphpad Prism v5.03 and IBM SPSS Statistics 23 software. A significance level of 0.05 was used for all hypothesis tests. Censor date was the 1st October 2017.

Results

Baseline demographics of the cohort are listed in Table 7. Median age was 68 years (40-89) with a M:F ratio of 1:1. Median serum creatinine was 98 µmol/L (32-610), eGFR 63 ml/min/1.73 m^2 (10-100) and NT-proBNP 2340 ng/L (12-42209). Median intraventricular septal thickness (IVSd) by echocardiography was 13 mm (7-22) with median left ventricular ejection fraction (LVEF) of 60% (20-77). 208/300 (69%) patients had renal involvement at baseline, 196/300 (65%) cardiac involvement, and 49/300 (16%) liver involvement. Visceral amyloid was evident by SAP scintigraphy in 237/300 (80%) patients; 72/237 (30%) had a large visceral amyloid load, 76/237 (32%) had a moderate visceral amyloid load and 89/237 (38%) had a small visceral amyloid load, as previously described.(65) Median survival by Kaplan Meier analysis was not reached although median time to death among 70/300 (23%) patients who died was only 3.4 (range 0.3-9.3) months.

Two hundred and eighty-two (94%) patients underwent BIA at baseline. Eighteen patients were too unwell to complete BIA analysis, 17 of whom had advanced cardiac amyloidosis (Mayo Stage 3b disease) and one of whom was unable to stand due to hypotension from amyloid-related autonomic neuropathy. One hundred and twenty patients underwent BIA at first follow

up, six months from baseline. The drop off in patient numbers at follow up was due to patient death in 70 cases; the remainder had not completed 6 months of follow up at the time of censor.

Table 7 Patient demographics and bioimpedence results at diagnosis

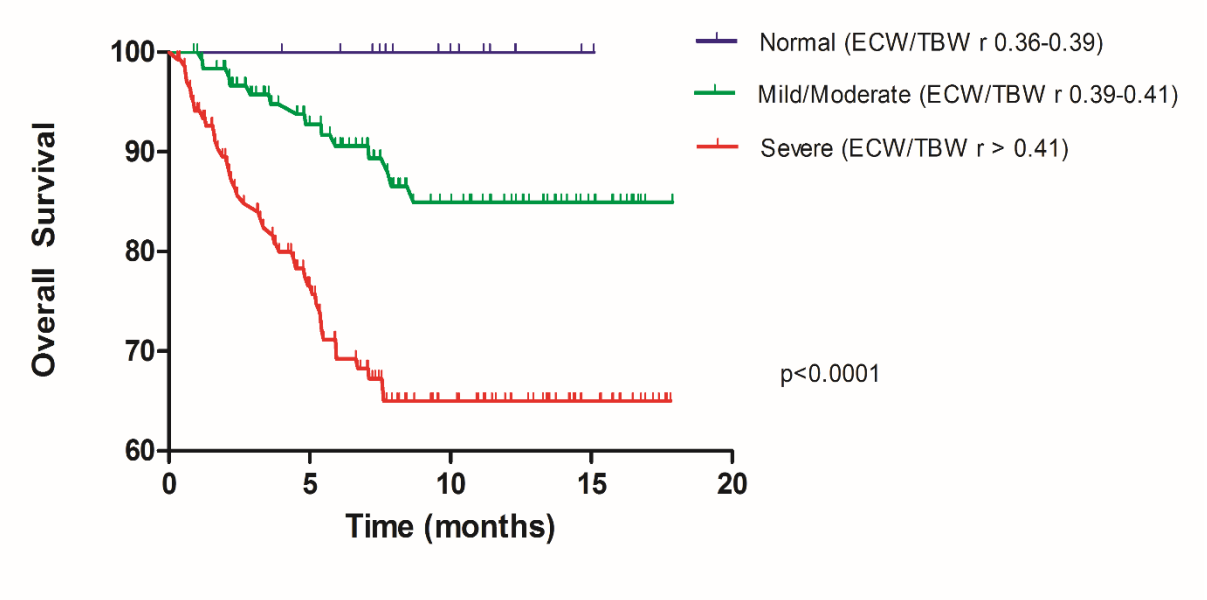
Demographics	**All**	**Male**	**Female**	**P value**
Sex		174 (58%)	126 (42%)	
Median Age (years) (Range)	68 (40-89)			
Median eGFR (ml/min) (Range)	63 (10-100)			
Median serum creatinine (µmol/L) (Range)	98 (32-610)			
Median serum Albumin (g/L) (Range)	34 (14-53)			
Median C-Reactive Protein (mg/L) (Range)	3 (<1-137)			
Visceral amyloid burden by SAP scintigraphy				
Large	72 (30%)			
Moderate	76 (32%)			
Small	89 (38%)			
None	63 (20%)			
Median IVSd (mm) (Range)	13 (7-22)			
Median LVEF (%) (Range)	60 (20-77)			
Median LVPW (mm) (Range)	13 (7-21)			
Median NT-proBNP (ngl/L) (Range)	2340 (12-42,209)			
Median 6MWT (metres) (Range)	374 (19-708)			
Mayo Disease Stage				
1	48 (16%)			
2	87 (29%)			
3	165 (55%)			
Bioimpedance				
Extracellular Water (ECW) (Litres) (Range)	17.1 (9.5-32.4)			
Total Body Water (TBW) (Litres) (Range)	41.4 (23.6-74.0)			
Intracellular Water (ICW) (Litres) (Range)	24.4 (14.0-41.6)			
ECW/TBW ratio (Range)	0.410 (0.366-0.450)			
ECW/TBW Median (range)				
Mayo Stage 1	0.401 (0.386-0.439)			
Mayo Stage 2	0.406 (0.379-0.450)			
Mayo Stage 3	0.416 (0.366-0.446)			<0.0001
Weight (kg) (Range)		82 (50.6-125)	67 (39.1-107.8)	
Body Mass Index (BMI) (Range)		27.2 (17.4-38.4)	25.5 (16.8-41.6)	
Fat Free Mass (kg) (Range)		65.4 (46.3-99.6)	46.7 (32.2-66.9)	
Skeletal Muscle Mass (SMM) (kg) (Range)		34.9 (24.3-52.2)	24.1 (16.3-34.3)	
Skeletal Muscle Mass/h^2 (Range)		11.3 (8.5-14.6)	9.51 (6.8-12.75)	
SMM (kg) Median (Range)				
Mayo Stage 1		39.05 (28.9-46.2)	24 (19.3-30.1)	
Mayo Stage 2		35.05 (28-52.20)	23.95 (16.3-32.7)	
Mayo Stage 3		34.60 (25-34.60)	24.7 (16.6-32.7)	0.1024
Body Fat Mass (kg) (Range)		16.8 (2.3-48.9)	20.5 (1.5-46.9)	
Body Fat Mass (kg) Median (Range)				
Mayo Stage 1		25.25 (5.9-45.1)	21.9 (7.4-39.8)	
Mayo Stage 2		18.95 (2.6-41)	21.85 (9.4-22.7)	
Mayo Stage 3		15.7 (2.6-35.10)	15.25 (1.5-42.1)	p=0.0008
Percentage Body Fat (%) (Range)		21 (3-40)	31 (3-49)	

IVSd – Interventricular septal diameter; LVPW - Left Ventricular Posterior Wall; 6MWT - Six minute walk test; ECW/TBW ratio – extracellular water/total body water ratio.

Extracellular water, Intracellular water and Total body water at baseline

At baseline; Median ECW, ICW and TBW were 17.1 L, 24.4 L and 41.4 L respectively. Median ECW/TBW ratio was 0.410 (normal range 0.360-0.390). Two hundred and sixty-four of 282 (94%) patients had ECW/TBW ratio above the normal range. The degree of fluid overload at baseline was a highly significant predictor of overall survival ($p<0.0001$) (Figure 7.1). There was a weak but significant correlation between baseline Log NT-proBNP concentration and ECW/TBW ratio (R^2 0.1139, $p<0.001$). ECW/TBW ratio ((R^2 0.098, $p<0.0001$) differed significantly between Mayo Disease Stages (Table 7).

Figure 7.1. Patient survival by Kaplan Meier stratified according to degree of ECV overload at the time of diagnosis. Patients were categorised as follows; normal ECV (ECW/TBW ratio 0.36-0.39), mild/moderate ECV overload (ECW/TBW ratio 0.39-0.41) and severe ECV overload (ECW/TBW >0.41) ($p<0.0001$).



Weight, Skeletal muscle mass and body fat mass at baseline

Male and female baseline skeletal muscle mass and body fat mass calculations are listed in Table 7. Median age in the male cohort was 68 years and median weight 82 kg. Median skeletal muscle mass (SMM) was 34.9 kg, median body fat mass (BFM) was 16.8 kg with a median percentage body fat (%BF) of 21% (normal range 10-20%). Median age in the female cohort was 69 years and median weight 67 kg. Median SMM was 24.1 kg, median BFM was 20.5 kg and median %BF was 31% (normal range 18-28%). SMM/h^2 revealed that no patient had severe sarcopenia at baseline, moderate sarcopenia was present in 53 (19%) patients and 227 (81%) patients had normal muscle mass. BFM (R^2 0.1330, p=0.0008) but not SMM differed significantly between Mayo Disease Stages.

BIA at first follow up (6 months from baseline)

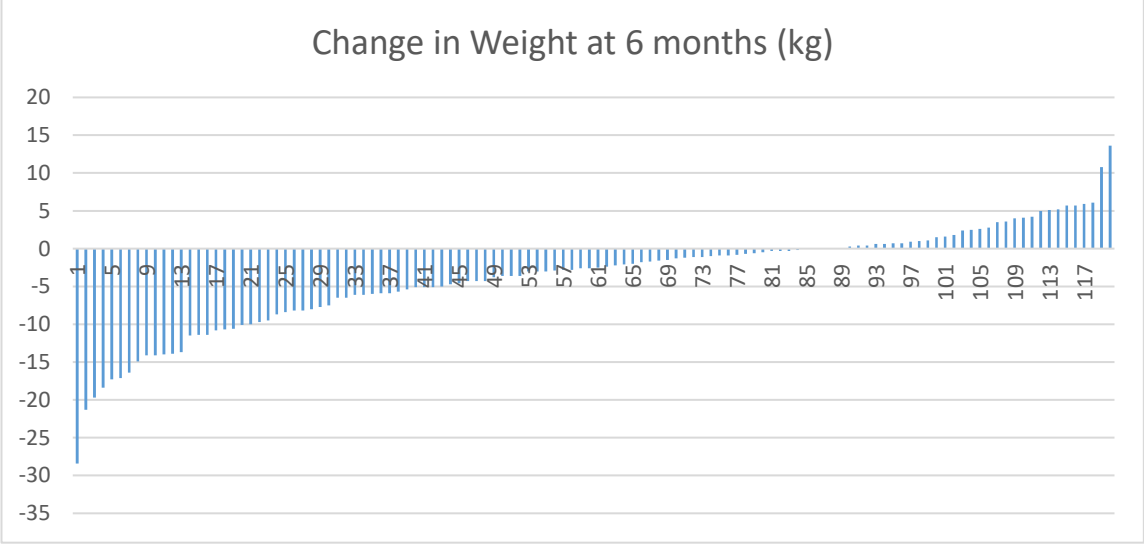
Waterfall plots of change in weight, ECW/TBW and SMM at 6 months from baseline are shown in Figure 7.2 (a, b and c respectively). BIA revealed an absolute median weight change at first follow up of -2.8 kg (range: -28.4 to +13.6), comprising a median loss of total body weight of 4%. Median ECW/TBW ratio change was + 0.005 (-0.04 to +0.05), representing a 2% median increase in ECW/TBW ratio. ECW/TBW increased in 77/120 patients, and remained the same or decreased in 43/120; the median increase in ECW/TBW ratio among the 77 patients in whom it increased was 0.01, equivalent to approximately 1.8 litres. Median change in SMM was -0.9 kg (range: -13.2 to +3.6). SMM decreased in 76/120 patients and remained the same or increased in 44/120 patients; median loss of SMM in the 76/120 patients in whom it decreased was 2kg. Follow up SMM/h^2 revealed development of severe sarcopenia in 2/120 (2%), moderate sarcopenia in 25/120 (22%) and normal muscle mass in 85/120 (76%) patients respectively. Median change in BFM was -2kg (range -13.7-8.4). BFM decreased in 75/120 patients and remained the same or increased in 45/120 patients; median loss of BFM in the 75 patients in whom it was decreased was 3.9kg.

There was a weak correlation between percentage change in ECW/TBW ratio and percentage change in Log NT-proBNP at 6 months (R^2 0.0852, p=0.008). There was no

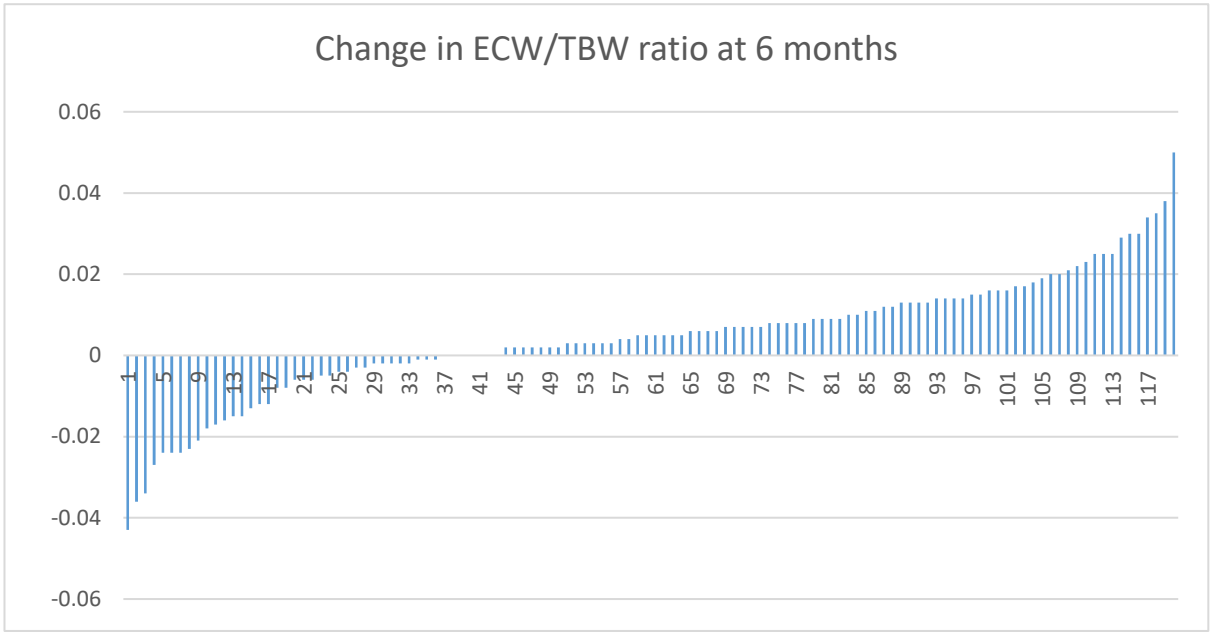
association between number of cycles of chemotherapy administered and change in ECW/TBW (p=0.906) or SMM (p=0.875). However, failing to achieve a dFLC response of ≥ 90% at 6 months was associated with an increase in ECW/TBW ratio (p=0.006). There was a reasonable correlation between change in body weight and change in ECW/TBW (R^2 0.1011, p=0.006) but a far stronger correlation between change in body weight and SMM (R^2 0.5548, p<0.0001).

Figure 7.2 Waterfall plot of change in BIA parameters at 6 months from baseline. A) Weight; B) Extracellular water/total body water (ECW/TBW) ratio; C) Skeletal muscle mass (SMM).

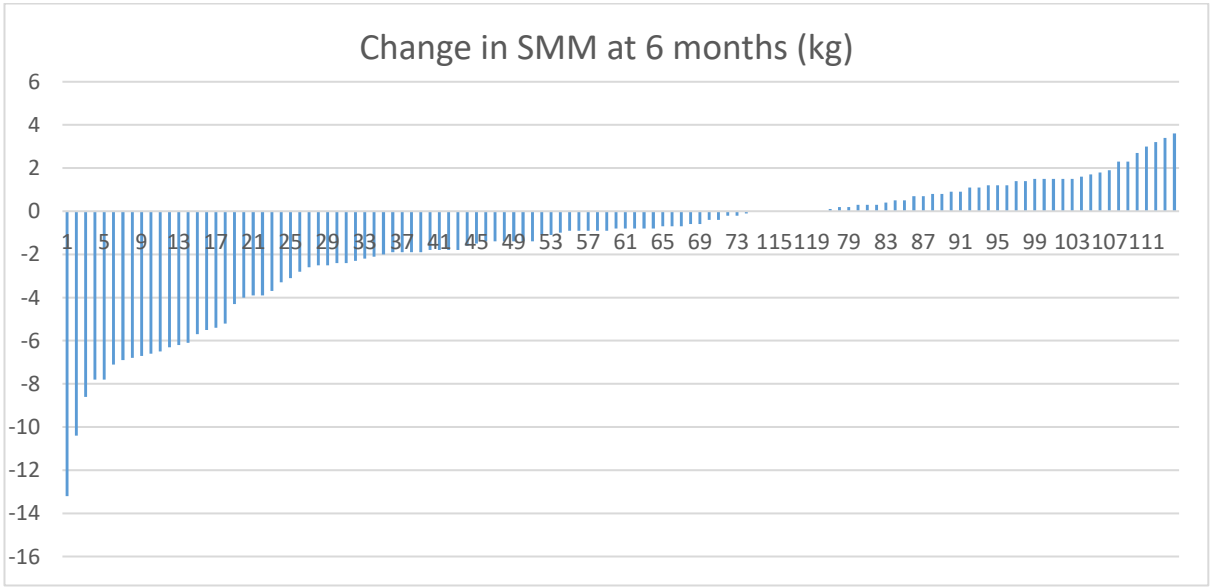
a)



b)


Change in ECW/TBW ratio at 6 months
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
101
105
109
113
117

c)


Change in SMM at 6 months (kg)
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
115
119
79
83
87
91
95
99
103
107
111

Discussion

Current management of fluid balance in AL amyloidosis is predominantly through clinical assessment and weight measurement despite their established limitations in diseases associated with sarcopenia.(175) Here we show for the first time that, prior to receiving systemic chemotherapy, ~95% of patients with systemic AL amyloidosis have significant fluid retention and ~20% of patients are sarcopenic. Additionally for the first time we show that the presence and degree of ECV overload (as defined by ECW/TBW ratio) at diagnosis is highly predictive of overall survival in patients with systemic AL amyloidosis.

Despite an overall loss of weight of 4% between baseline and follow up, there was an increase rather than a reduction in ECV overload in the majority of patients. Median loss of SMM and BFM was 1 kg and 2 kg respectively and this was accompanied by an increased incidence of sarcopenia. These data highlight that measurement of weight alone in patients with AL amyloidosis receiving chemotherapy is likely to underestimate loss of SMM due to worsening co-existing volume overload and that weight loss is often due to loss of SMM and BFM rather than adequate diuresis. The commonest reason for acute admission to hospital during chemotherapy among UK AL amyloidosis patients is fluid retention (unpublished) which ought to be preventable but is entirely consistent with the present findings. Serial BIA measurements, by identifying progressive fluid retention despite loss of muscle mass, would enable targeted management of fluid balance as well as nutritional status.

BIA measurements in patients with systemic AL amyloidosis revealed a correlation, albeit relatively weak, between baseline NT-proBNP and ECW/TBW ratio and also between change in NT-proBNP and change in ECW/TBW ratio. This correlation has previously been shown in dialysis cohorts where BIA is known to aid assessment and management of volume status (175). NT-proBNP is a key prognostic biomarker in patients with systemic AL amyloidosis, usually indicating presence and severity of cardiac infiltration;(101) however, here we show for the first time the association between degree of ECV overload and patient survival. Marked fluctuations in NT-proBNP concentration occur, particularly during chemotherapy and

our findings highlight the need for further work to better understand the precise relationship between BIA assessment of ECV overload and NT-proBNP in patients with AL amyloidosis.

Interestingly, achieving less than a hematologic very good partial response (VGPR) was associated with an almost 8 fold increase in mean ECW/TBW change compared to achieving a VGPR or CR. It is well established that achieving a hematologic VGPR or CR is prognostically beneficial in AL amyloidosis,(68) and one might postulate that the worsening fluid retention in patients who fail to achieve adequate clonal responses is directly related to worsening amyloidotic organ dysfunction.

Treatment of systemic AL amyloidosis is associated with major morbidity and occasionally, mortality. Sarcopenia is an increasingly recognised independent risk factor for treatment intolerance,(179) and fluid retention commonly prompts emergency hospital admission among AL amyloidosis patients receiving chemotherapy. BIA may enable early recognition and intervention of changes in body composition during chemotherapy. Further work to assess the role of BIA in predicting and influencing clinical outcomes in AL amyloidosis is warranted.

Chapter Eight: Role of implantable intracardiac defibrillators in patients with cardiac immunoglobulin light chain amyloidosis

Introduction

Immunoglobulin associated light chain (AL) amyloidosis is a rare clonal plasma cell disorder characterized by the deposition of misfolded amyloid proteins in vital organs including the kidneys and heart(5). Over half of patients present with cardiac involvement and prognosis is predominantly dictated by the presence and severity of cardiac involvement (180). Severity of cardiac organ involvement is assessed by serum biomarkers, echocardiography and increasingly cardiac magnetic resonance (CMR)(72). One third of patients with cardiac AL amyloidosis present with a significant elevation of both N-terminal pro-brain natriuretic peptide (NT-proBNP) and troponin T (TnT) concentration (68) and are classified according to the widely used Mayo Clinic criteria (86) to have Stage III disease which is associated with a poor overall survival (median of 9-12 months)(181).

The particular challenge in systemic AL amyloidosis is a very high early mortality, often within the first few months of diagnosis (30-40%), despite significant improvement in treatment regimes(75). In addition, only 29% of those with very advanced cardiac AL survive longer than 12 months from diagnosis (68, 182). The main cause of this significant and early mortality is sudden cardiac death (SCD) – the trigger (and consequently treatment) of which remains unclear. Whilst the prevalence of ventricular arrhythmias is as high as 27% in patients with cardiac AL amyloidosis(76), both ventricular tachyarrhythmias(77, 183) and bradycardia (78) have been observed as terminal events. This lends to ICD implantation being a logical therapeutic intervention but efficacy, criteria for patient selection and more importantly long term survival benefit have yet to be determined. To date only one study has suggested a long term survival benefit of ICD implantation in a select cohort of patients with cardiac amyloidosis (184).

In this study, we report the UK experience with ICD implantation in a cohort of patients with cardiac AL amyloidosis managed by a uniform supportive care and treatment pathway.

Methods

This study included all newly diagnosed patients with systemic AL amyloidosis who had an ICD implantation as part of the treatment plan and managed according to our protocolised multidisciplinary treatment pathway at the Royal Free Amyloidosis treatment centre from June 2010 to November 2015. The diagnosis of amyloidosis was confirmed in all cases with a tissue biopsy demonstrating characteristic birefringence on Congo red staining viewed under cross polarized light. Typing of AL amyloidosis was confirmed by immunohistochemical staining with appropriate antibodies to kappa or lambda light chains and by exclusion of hereditary amyloidosis, where necessary, by genetic sequencing of the relevant genes.

All patients underwent a comprehensive clinical consultation, evaluation at baseline and then subsequently every six months in a protocolised manner including electrocardiogram (ECG), transthoracic echocardiogram including tissue Doppler imaging, whole body ^{123}I-serum amyloid P component scintigraphy (SAP scan)(185), standardized 6 minute walk test, lying and standing blood pressure measurements, New York Heart Association (NYHA) and Eastern Cooperative Group (ECOG) Grading. Clonal disease was assessed serologically by serial identification and quantitation of any circulating monoclonal protein and serum free light chains (Freelite™ assay [The Binding site, Birmingham, United Kingdom]) measured monthly. Conduction abnormalities on ECG were defined as per standard accepted criteria(186, 187). Detailed echocardiogram included assessment of diastolic dysfunction, 2-D left ventricular strain and tissue Doppler imaging were performed as previously described (188). Organ involvement and hematological responses were defined as per the international amyloidosis consensus criteria (69). Cardiac disease stage was defined as per the criteria described (86). Additionally, patients with Mayo cardiac stage III disease were divided as stage IIIa if the NT-proBNP was <8500 ng/L and stage IIIb if NT-proBNP was ≥8500 ng/L(68, 160).

Chemotherapy treatment was undertaken as per the UK amyloidosis treatment guidelines (178). As a precautionary measure, due to the known high early mortality, most patients with stage III cardiac amyloidosis in the UK are admitted for 48-72 hours of continuous ECG monitoring at the start of chemotherapy typically starting at least 24 hours before the first chemotherapy dose. ICD implantation was considered as per the UK guidelines (https://www.nice.org.uk/guidance/ta314/evidence). Serious recurrent ventricular arrhythmias defined as non-sustained ventricular tachycardia on more than one occasion in the presence of syncope/presyncope or sustained VT were considered for ICD implantation. Patients undertook routine downloads from their devices on a weekly basis and additional download at the time of symptoms. These were reviewed by the ICD team including a cardiologist.

Where relevant, time to death was recorded and every effort was made to obtain clinical details and ICD 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. Censor date for surviving patients was 1 January 2016.

All patients were treated in accordance with the Declaration of Helsinki and provided informed consent for insertion of the ICD and publication of data.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 statistical software package. Estimated median patient survival was calculated by Kaplan-Meir analysis using GraphPad Prism v5.03 software.

Results

Patients

A total of 15 patients who underwent ICD implantation from 2010 to 2015 were identified. Baseline characteristics are found in Table 8.1. Median age at diagnosis was 51 years (range 37-80) M: F ratio 3:2. Median serum high-sensitivity TnT was 60ng/L (range 22-326 ng/L) and median NT-proBNP concentration was 5178 ng/L (range 2220 – 34,153). Mayo Staging II, IIIa and IIIb were 4/15, 8/15 and 3/15 respectively. One patient had Mayo stage II disease but an NT-proBNP >8500 ng/mL. All patients had cardiac involvement with renal, liver and nerve involvement in 7/15 (47 %%), 5/15 (33%) and 1/15 (7%) patients respectively. At presentation, five patients were receiving β-blocker therapy. No patients were receiving amiodarone or other antiarrhythmics. All patients received chemotherapy as per standard UK treatment guidelines with front line Bortezomib or Thalidomide based regime.

The baseline electrocardiogram showed that: 13 (87%) patients were in sinus rhythm. 2 (13%) had atrial fibrillation. Only one patient had evidence of 1st degree atrio-ventricular conduction block (normal PR interval in 12 of the 13 patients in sinus rhythm). There was intraventricular conduction delay in 7 (46 %) patients – right bundle branch block in 1/7 (14%) and non-specific intraventricular conduction defects with prolonged QRS in 6/7 (84%). On echocardiography, the median left ventricular wall thickness was 15mm (range 12-19mm). On categorical scoring, the diastolic dysfunction was mild, moderate and severe in 2 (13%), 6 (40%) and 7 (47%), respectively. Median global left ventricular strain was −9.5% (range −0.08 to −18.2%) and median left ventricular ejection fraction (LVEF) was 53% (range 40-65 %).

Table 8.1. Baseline Demographics in ICD cohort

Demographic	**N (%) or Median (Range)**
Male: Female	9 (60%):6 (40%)
Age (years)	51(37-80)
Systolic BP (mmHg)	110 (79-133)
Diastolic BP (mmHg)	68 (29-83)
NYHA class • 1 or 2 • 3	 14 (93%) 1 (7%)
6 Minute walk distance (metres)	370 (138-607)
Clonal disease markers	
Paraprotein present • IgG	8 (53%) 8 (100%)
Serum Free light chains	
Kappa (n) Starting light chains (mg/L)	4 610 (319-690)
Lambda (n) Starting light chains (mg/L)	11 418 (82-3260)
dFLC (mg/L)	482 (60 -3245)
Cardiac Markers:	
High sensitivity Troponin T (ug/L)	60 (22-326)
NT pro-BNP (ng/L)	5178 (2220-34,153)
Echocardiographic parameters: • LVEF by Biplane Simpson (%) • Septal wall thickness (mm) • Global LV strain by tissue Doppler imaging.	 53 (40-65) 15 (12-19) -9.5% (-0.08 to – 18.2%)
ECG parameters: • Sinus rythmn • Atrial Fibrillation • PR interval (ms) • QRS interval (ms) • QTc duration (ms)	 13 (87%) 2 (13%) 164 (138-242) 98 (84-162) 481 (401-520)
Mayo Disease Stage • 2 • 3a • 3b	 4 (27%) 8 (53%) 3 (20%)
Organ involvement (Liver, Kidney, Spleen, Nerve) • Liver • Kidney • Spleen • Nerve	 5 (33%) 7 (47%) 8 (53%) 1 (7%)
Renin Angiotensin Blockade	5 (33%)
Diuretic treatment • Loop diuretic • Mineralocorticoid receptor antagonist	 11 (73%) 5 (33%)

ICD implantation and arrhythmias

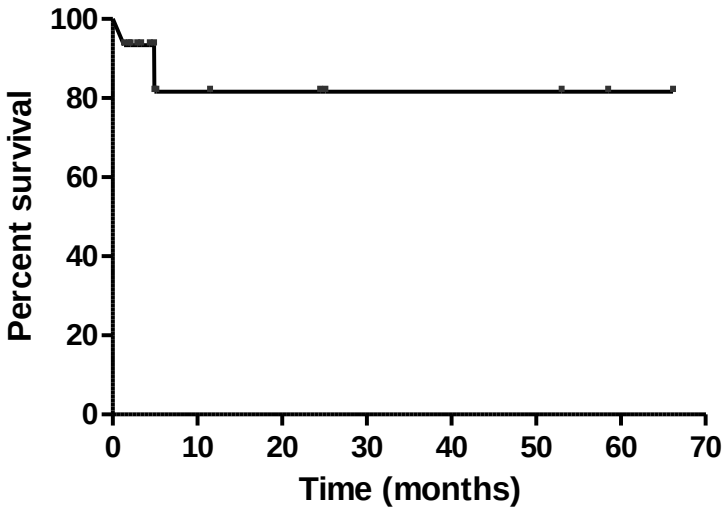
Median time to ICD implantation was 13 days from their first presentation to the NAC and median follow-up from diagnosis was 4.9 months (range 1.35 – 66.12). All arrhythmias were detected by continuous cardiac monitoring when patients were admitted for assessment prior to starting chemotherapy as per our standard protocol. Only 4 (27%) patients gave a history of recurrent syncope. Fourteen patients (93%) underwent ICD implantation for primary prevention and only one patient underwent implantation for secondary prevention after a cardiac arrest due to ventricular fibrillation. The underlying arrhythmia in the 14 patients who underwent ICD implantation for primary prevention were: non-sustained ventricular tachycardia in 12 patients (80%) and sustained ventricular tachycardia with spontaneous reversion in 2 (13%).

13/15 (87%) of the patients were started on oral amiodarone after ICD implantation. The remaining 2 patients received beta blocker therapy (one of whom was intolerant of amiodarone). All patients routinely downloaded their data via a modem or had their ICD routinely assessed for the presence of arrhythmia after implantation. The presence of NSVT was detected in 6 (40%) patients, of which 5/6 (83%) were on amiodarone therapy and 1/6 (17%) was receiving beta blocker therapy.

Appropriate ICD device therapy and impact on survival

A total of four patients had appropriate therapy from their ICD. Three (20%) patients received appropriate therapy from their ICD for ventricular arrhythmias and one patient had pacing for bradyarrhythmia. Overall, 13 out of 15 patients are alive with overall survival at 3, 6 and 12 months of 93%, 82% and 82% respectively (Figure 8.1). Two out of the four patients who had appropriate ICD therapy from their device have since died.

Figure 8.1. Overall survival in cohort by Kaplan Meir Analysis. Overall survival at 3, 6 and 12 months was 93%, 82% and 82% respectively.

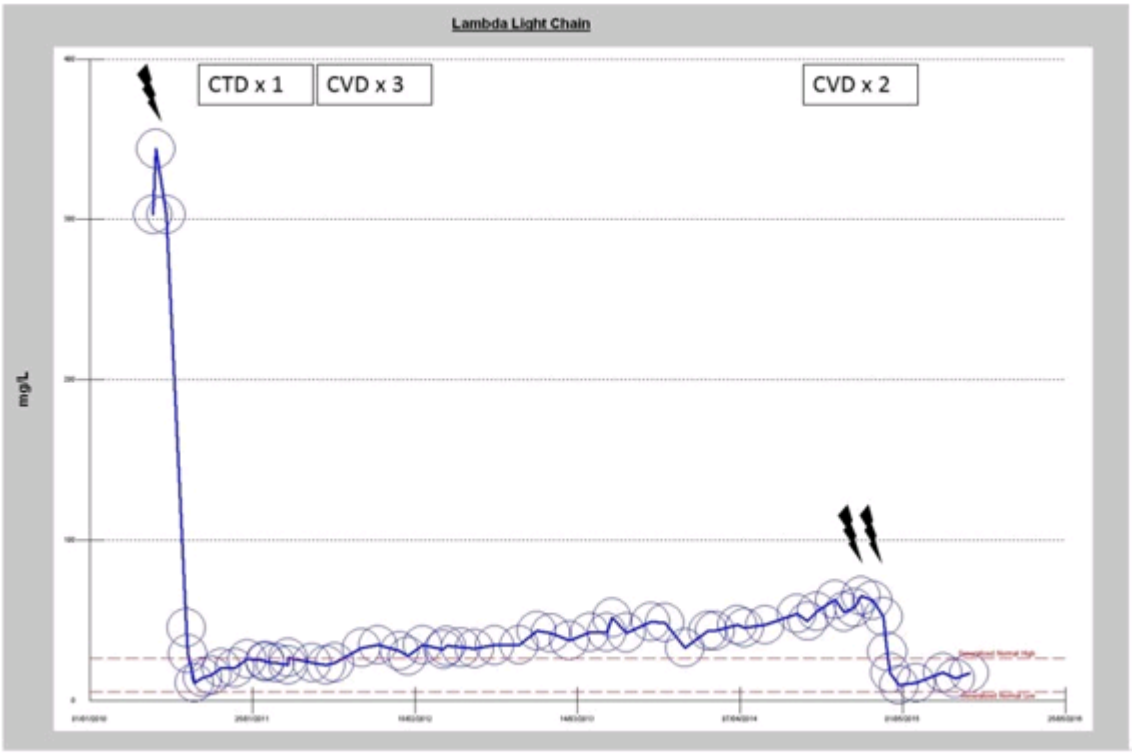


Patient 1 (Mayo Stage 2) developed VF and had antitachycardia pacing followed by a single 41J shock. The patient suffered no ill effects. This patient is still alive 41 months after having achieved a complete response with a Bortezomib based treatment. This patient had a subtle clonal relapse and had two further episodes of VF needing two shocks – both successfully reverting her back to sinus rhythm. Figure 8.2 shows this patient's clinical course. Patient 2 (Mayo Stage 2 with NT-proBNP >8500ng/L) developed VF during the first cycle of chemotherapy within 8 days of commencing treatment and successfully reverted to sinus rhythm. However, ten days later, the patient had further VF which was appropriately shocked but was intractable and the patient died. There was no evidence of a clonal response at the time of death. Patient number 3 (Mayo stage 3b disease) developed VF during her 2nd cycle of chemotherapy and was also appropriately shocked. She remained in sinus rhythm after the shock until death. She had refractory clonal disease and died over 3 months later with end stage heart failure.

Patient 4 who underwent ICD implantation for NSVT, developed marked bradycardia with symptomatic hypotension/syncope and required pacing. The lower pacing threshold was increased from 40 bpm to 60 bpm and the symptoms resolved.

Figure 8.2

Graph showing (patient 1) lambda light chains from diagnosis, chemotherapy with Cyclophosphamide Thalidomide and Dexamethasone (CTD), Cyclophosphamide Velcade and Dexamethasone (CVD) and the number of cycles. Arrows indicate the time point of appropriate ICD therapy with 41J shock.



Discussion

Survival in systemic AL amyloidosis has substantially improved over the last two decades, however early mortality has essentially remained unchanged with SCD almost always the cause of death. With the increasing use of continuous electrocardiographic monitoring, arrhythmias are frequently detected in cardiac AL amyloidosis. There is no clear consensus on either the best immediate treatment or indeed long term management. ICD implantation is a potentially attractive option addressing both the ventricular tachyarrhythmias and bradycardias. Studies reporting the outcomes of ICD implantation in cardiac amyloidosis remain limited and conflicting. We report a quarter of the patients in the current cohort received appropriate therapy from the ICD, an additional third of patients had NSVT which did not need ICD intervention and a good overall survival of 82% at 12 months. In the immediate short term, ICD was life-saving in all cases who received appropriate ICD therapy but only two out of the four patients achieved longer term survival. One patient died prior to completion of their first cycle of chemotherapy (before a clonal response could be achieved) and the second was a non-responder to chemotherapy (a very poor prognostic parameter)(189).

The key to improved outcomes in AL amyloidosis is achieving a very good free light chain response to therapy along with a reduction in cardiac biomarkers which heralds an end organ response(101). However, a third of the patients succumb to cardiac death before such a response can be achieved. Attempts to reduce this early mortality have varied from admission for cardiac monitoring during the early cycles (standard UK practice), use of anti-arrhythmic therapy(190) and dose modified chemotherapy to reduce the potential risk of death attributable to toxic adverse effects. Small molecules which block light chain mediated cardiac toxicity such as doxycycline(191) or Epigallocatechin-3-gallate (EGCG)(192) have been attractive agents but compelling prospective data on their activity is not available.

ICDs have a clear role in improving outcomes in systolic heart failure and arrhythmogenic disorders such as hypertrophic cardiomyopathy but not in non-ischemic heart failure(193). As cardiac arrhythmias are supposedly the main cause of early death in cardiac AL amyloidosis, ICD implantation is a logical but unproven solution. Assessing the impact of ICD implantation in

cardiac AL amyloidosis focuses on two separate issues - the appropriateness of ICD therapy in terminating a life threatening cardiac arrhythmia (i.e. improvement in immediate survival) and its role in improving long term survival (Table 8.2). A study from the German amyloidosis group failed to demonstrate any benefit with a high mortality of 47% - including appropriate shock therapy in only 2 patients (11%) and only one patient surviving post therapy (194). Contrarily, two other studies from the US, reported appropriate shock therapy in just over a third of patients (184, 195), data which is similar to our current results. In the current series, all four patients with serious arrhythmias post ICD implantation had appropriate therapy – similar to reports from the two US series with up to 80% immediate survival post ICD therapy. In our series, the long term impact is challenging to interpret due to the small number of events – clearly there was no impact on long term survival in two patients and was life prolonging in the longer term for the remaining two patients.

Meta-analysis of the data available (including our cohort) contains 82 reported patients with AL amyloidosis (Table 8.2). It is clear that patients with cardiac AL amyloidosis have a high burden of ventricular arrhythmias and the majority of ICD implantation was for primary prevention. Only 28% of patients actually required device therapy. Importantly, nearly three quarters of all patients who had therapy from their ICD survived immediately following the therapy. This is a crucial point – in patients with AL amyloidosis, survival in the early months is important to allow delivery of chemotherapy which will reduce the light chain burden and hence, potentially allow for benefit from treatment and longer term survival. Only half of all patients reported, including the current series, survived long term following the ICD therapy. This data would suggest that, in appropriately selected patients with cardiac AL amyloidosis, ICDs will deliver appropriate therapy that is lifesaving, in the short term, in the majority of patients. Nonetheless, other factors including initial amyloid burden, clonal response, toxicity from chemotherapy and concurrent vital organ involvement, are likely determinants of the longer term outcomes and not impacted by ICDs. Based on this, we propose an algorithm for consideration of ICD implantation in patients with AL amyloidosis (Figure 8.3).

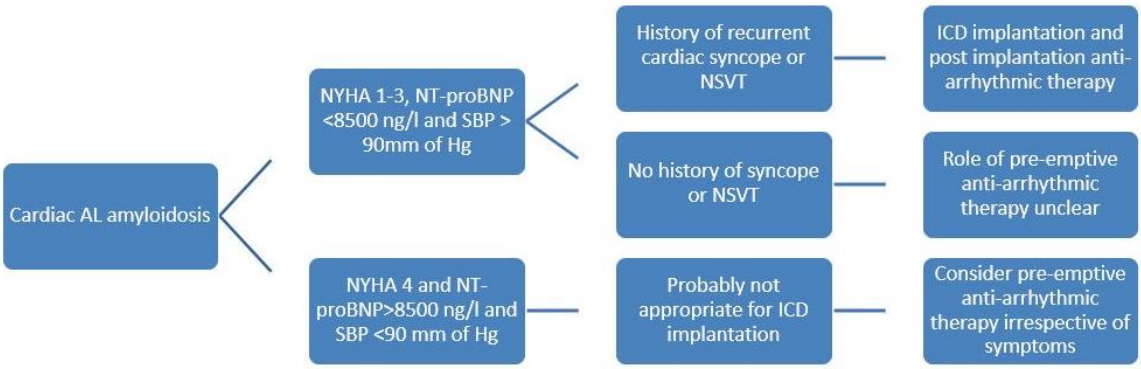
Table 8.2. Meta-analysis of studies to date on ICD implantation in patients with cardiac amyloidosis.

Study/Date	Patients with AL	Primary/ secondary prevention	Appropriate therapy from device (%)	Survival post therapy (%)	Survival during follow up of patients who received appropriate therapy (%)	Overall survival for cohort
Varr et al (2014)	15	11 Primary prevention 4 Secondary prevention	5/15 (33%)	4/5 (80%)	1/4 (25%)	
Lin G et al (2013)	33	25 Primary prevention 8 Secondary prevention	12/33 (36%)			10/33 (30%)
Kristen et al (2008)	19	19 Primary prevention	2/19 (11%)	1/2 (50%)	1/2 (50%)	10/19 (53%)
Current series	15	14 Primary prevention 1 Secondary prevention	4/15 (27%)	3/4 (75%)	2/4 (50%)	13/15 (87%)
ALL Studies	82	69 Primary prevention 13 Secondary prevention	23/82 (28%)	8/11 (73%)	4/10 (40%)	33/67 (49%)

Limitations of our study include patient selection, which was restricted to those treated at our centre. Alongside this the majority of our patients were on routine amiodarone after ICD implantation although amiodarone has not been shown to be better than placebo with regards to survival in congestive heart failure (196). Finally our sample size was small, limiting our ability to interpret sub-group data.

In conclusion, ICDs deliver appropriate therapy in the majority of patients with cardiac AL amyloidosis – although lifesaving in the short term, their long term survival benefit remains unclear. Appropriate patient selection is crucial to the rationalized use of scarce resources and patients with moderate cardiac amyloidosis, who are likely to benefit most, should be considered. Efforts directed at the prevention of arrhythmias may be more appropriate for the most advanced disease (Mayo stage IIIb). Formal prospective studies of device therapy in AL amyloidosis is urgently needed to confirm the validity of this approach.

Figure 8.3: Approach to ICD implantation in cardiac AL amyloidosis.



Chapter Nine: General Conclusions

The studies presented in this thesis address key areas of diagnosis, monitoring and outcome of patients with cardiac and renal amyloidosis. Definitive diagnosis of amyloidosis and confirmation of fibril protein type, along with evaluation of vital organ involvement, are critical for optimal clinical management. I have characterized the importance of using both CR and IHC staining alongside LDMS to correctly identify both the presence and type of amyloid deposits across a range of different tissues. The strength of IHC staining and limitations of mass spectrometry in renal tissue is demonstrated in our series of patients with ALECT2 amyloidosis, in which LDMS is complimentary but not superior to IHC. The ALECT2 study also provides novel information on the prevalence of this non-hereditary and slowly progressive form of renal amyloidosis, describing a more distinct ethnic preponderance than previously noted; unusually, renal ALECT2 amyloidosis does not present with classical nephrotic range proteinuria. Its association with hepatitis infection and a potential state of low grade chronic inflammation offers potential insight into the pathogenesis of this curious disease.

The management of systemic AL amyloidosis is influenced strongly by assessment of individual organ involvement despite it being a complex multisystem disease in which the various involved organ systems must necessarily affect others. To date, management of renal AL amyloidosis with advanced renal excretory impairment at presentation has focused upon preserving renal function and in clinical practice this has led to a reluctance to deliver systemic chemotherapy to patients deemed at ‘high risk’ of reaching RRT. As part of our work, here we show, for the first time, that in patients with systemic AL amyloidosis and advanced renal excretory impairment at presentation both the speed and depth of clonal response is crucial to protect against death, dialysis and their composite endpoint. Alongside this almost one third of patients with systemic AL amyloidosis have combined cardiac and renal organ dysfunction at presentation (Type 5 cardiorenal syndrome), and their outcomes are poor despite chemotherapy. Our study demonstrated that

nearly two thirds of the cohort died, among whom median time to death was 5 months and 16% developed ESRD, the majority of whom reached dialysis within 12 months of diagnosis. Our data is novel in that it concerns three hard outcome measures: death, dialysis and their composite endpoint. Following this work we propose a novel risk stratification tool, in patients with systemic AL amyloidosis and cardiorenal syndrome based upon cardiac (NT-proBNP) and renal (eGFR) biomarkers during the first 12 months following diagnosis.

Cardiac and renal AL amyloidosis often lead to both ECV overload and sarcopenia. Nutritional status both at diagnosis and throughout treatment has been shown to influence prognosis and quality of life respectively. Our study looking at the role of body composition using BIVA in patients with systemic AL amyloidosis demonstrated that 94% of patients at baseline had ECV overload and almost 20% had sarcopenia. The degree of fluid overload at baseline was highly predictive of overall survival and whilst there was a weak correlation between ECV overload and NT-proBNP, this study offerred some insight into the relationship between fluid status and NT-proBNP, which is a key predictor of mortality in systemic AL amyloidosis.

Cardiac involvement remains the main determinant of death in systemic AL amyloidosis. Whilst treatment options and chemotherapy responses have improved significantly over the past decade, sudden and early cardiac death remains common. Our work looking at the role of ICDs has shown that there is a significant incidence of ventricular arrhythmias in patients with cardiac AL amyloidosis and whilst ICD implantation offered appropriate and lifesaving therapy in a quarter of our cohort, there was no clear survival benefit. This seems to be particularly relevant to those patients with the most advanced disease (Mayo Stage IIIb) and in those cases, where ICD implantation may not offer a survival benefit, efforts should be directed at oral anti-arrhythmic therapy. Nonetheless in light of the high incidence of sudden cardiac death, importance of delivering early chemotherapy and the significant cost associated with ICD implantation, formal prospective studies are urgently needed to confirm the validity of this approach.

Appendix I – Future Work

The results of chapter 4, exploring the role of proteomics in the diagnosis and typing of amyloid deposits has demonstrated the superior role of proteomics in the typing of amyloidosis. Currently, target organ biopsies, typically of the heart or kidneys, are regarded to be the 'gold standard' for definitive histological diagnosis. Cardiac biopsies are not without risk, require specialist training and are available in a limited number of centres in the U.K, often leading to a delay in diagnosis. Fat aspiration is a simple, safe and quick bedside test, potentially allowing for a histological diagnosis without the need for a target organ biopsy. Following on from chapter 4, we will conduct a prospective study, comparing proteomic analysis of fat aspirates to both CR and IHC, and whether proteomic analysis of adipose tissue can improve the diagnosis and typing of systemic amyloidosis.

Renal involvement in AL amyloidosis is the commonest organ manifestation and the aim of treatment is to prevent worsening of renal function and the need for RRT. Chapter 5 has identified the importance of a rapid and deep clonal response in patients with systemic AL amyloidosis and advanced renal excretory function at presentation whilst Chapter 6 has helped risk stratify patients with combined renal and cardiac amyloidotic organ dysfunction at baseline. Nonetheless, kidneys infiltrated with amyloid are exquisitely vulnerable to intercurrent insults, often leading to AKI in CKD. The role of proximal tubular dysfunction (PTD) in renal AL amyloidosis is not clear however, plasma cell dyscrasias are known to cause PTD (92). Urinary retinol binding protein (uRBP), a low molecular weight protein which is freely filtered at the glomerulus and almost completely reabsorbed at the proximal tubule(197) is a well-recognized marker for proximal tubular dysfuction. We plan to study baseline uRBP levels in all patients with newly diagnosed systemic AL amyloidosis, referred to our national centre, prior to receiving systemic chemotherapy and its correlation with both biochemical and histological parameters as well as renal and overall survival.

The role of nutritional status and its effect on both morbidity and mortality in AL amyloidosis has been previously reported. Chapter 7 has identified the incidence of both

ECV overload and sarcopenia in patients with AL amyloidosis receiving systemic chemotherapy. We plan to expand on this work with a prospective randomized controlled trial comparing the role of supervised exercise and dietetic support compared to best clinical care during the first 12 months of chemotherapy and its effect on exercise tolerance (6MWT), body composition as assessed by BIVA, quality of life and survival in patients with systemic AL amyloidosis.

Appendix II - Publications Arising from Thesis Work

1. *Systemic Amyloidosis*. Rezk T, Hawkins PN, **Textbook of Autoinflammation**, Edition 15. **2019**

2. *The complementary role of histology and proteomics for diagnosis and typing of systemic amyloidosis*. Rezk T, Gilbertson JA, Mangione PP, Rowczenio D, Rendell N, Canetti D, Lachmann HJ, Wechalekar AD, Bass P, Hawkins PN, Bellotti V, Taylor GW, Gillmore JD. **J Pathol Clin Res 2019**

3. *Diagnosis, pathogenesis and outcome in leucocyte chemotactic factor 2 (ALECT2) amyloidosis.* Rezk T, Gilbertson JA, Rowczenio D, Bass P, Lachmann HJ, Wechalekar AD, Fontana M, Mahmood S, Sachchithanantham S, Whelan CJ, Wong J, Rendell N, Taylor GW, Hawkins PN, Gillmore JD. **NDT 2016**

4. *Prolonged renal survival in light chain amyloidosis: speed and magnitude of light chain reduction is the crucial factor.* Rezk T, Lachmann HJ, Fontana M, Sachchithanantham S, Mahmood S, Petrie A, Whelan CJ, Pinney JH, Foard D, Lane T, Youngstein T, Wechalekar AD, Bass P, Hawkins PN, Gillmore JD. **Kidney Int. 2017.**

5. *Cardiorenal AL amyloidosis: Risk stratification and outcomes based upon cardiac and renal biomarkers.* Rezk T, Lachmann HJ, Fontana M, Martinez De Azcona Naharro A, Sachchithanantham S, Mahmood S, Petrie A, Whelan CJ, Pinney JH, Foard D, Lane T, Youngstein T, Wechalekar AD, Bass P, Hawkins PN, Gillmore JD. **Br J Haematol. 2019.**

6. *Bioimpedance vector analysis for the detection of extra-cellular volume overload, a new prognostic biomarker, coupled with sarcopenia in systemic AL amyloidosis*. Rezk T, Davenport A, Gan JJ, Lachmann HJ, Fontana M, Martinez-Naharro A, Sachchithanantham S, Guillotte C, Mahmood S, Petrie A, Whelan CJ, Pinney JH, Foard D, Lane T, Youngstein T, Wechalekar AD, Hawkins PN, Gillmore JD. **Br J Haematol. 2018.**

7. *Role of implantable intracardiac defibrillators in patients with cardiac immunoglobulin light chain amyloidosis.* Rezk T, Whelan CJ, Lachmann HJ, Fontana M, Sachchithanantham S, Mahmood S, Khan F, Khiani R, Tomson J, Youngstein T, Gillmore JD, Hawkins PN, Wechalekar AD. **Br J Haematol. 2017.**

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